A PHYTOCHEMICAL INVESTIGATION OF THE TOXIC PLANT EUPATORIUM RUGOSUM

A THESIS

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By

Peter Edward May

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A PHYTOCHEMICAL INVESTIGATION OF THE TOXIC PLANT EUPATORIUM RUGOSUM

Approved:

Leon H. Zalkow, Chairman

C. L. Liotta

Erling Grovenstein, Jr.

Date approved by Chairman 11/13/78

TO

JIM AND ANN MAY

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Leon H. Zalkow for his guidance during the course of this work, and for his seemingly inexhaustible patience. I would also like to thank my parents, for their moral and financial support, and all of my co-workers in Dr. Zalkow's research group, whose assistance proved invaluable.

Finally I wish to convey my gratitute to Professors Charles Liotta and Erling Grovenstein for serving as my reading committee, and to the Chemistry Department for financial assistance in the form of a Teaching Assistantship (1976-1978).

TABLE OF CONTENTS

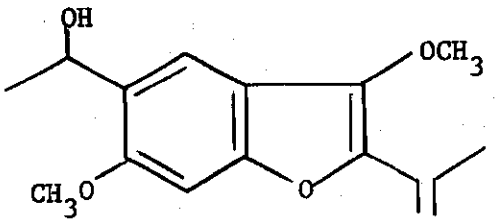
GLOSSARY OF ABBREVIATIONS

bs	broad singlet (NMR)
C	Celsius
cm^{-1}	wave number (IR)
col	column
d	doublet (NMR)
dd	doublet of doublets (NMR)
frn.	fraction
g	gram
GC	Gas Chromatography
HPLC	High Pressure Liquid Chromatography
Hz	Hertz (cycles per second)
IR	infrared spectroscopy
J	coupling constant (NMR)
kg	kilogram
l	liter
m	multiplet (NMR)
M^+	molecular ion in mass spectrum
m/e	mass to charge ratio (mass spectrum)
min	minute
mg	milligram
ml	milliliter
MP	melting point
nm	nanometer

NMR	nuclear magnetic resonance spectroscopy
p.	page
ppm	parts per million (NMR)
q	quartet (NMR)
R_+	retention time
s	singlet (NMR)
sec	seconds
t	triplet (NMR)
TMS	tetramethylsilane
UV	ultraviolet spectroscopy

SUMMARY

Investigation of an extract of the plant Eupatorium rugosum (white snakeroot) has resulted in the isolation and identification of two new benzofurans, rugosumol (V) and rugosumone (VI).



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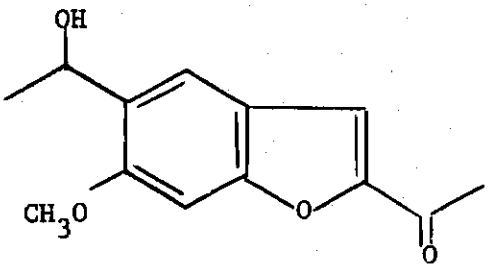
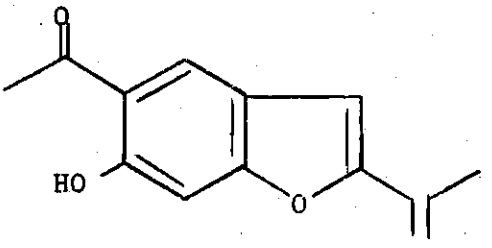


VI

The compounds phytol (VII), stigmosta-9,22-dien-3B-ol (VIII) and hydroxytremetone (III) were also isolated. Of these three compounds only hydroxytremetone (III) has been reported as occurring in the plant.



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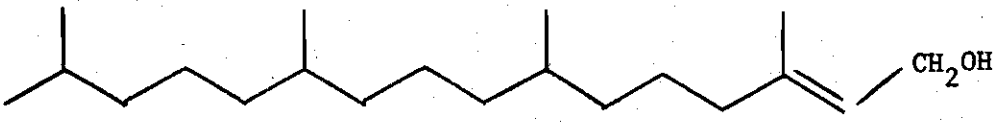

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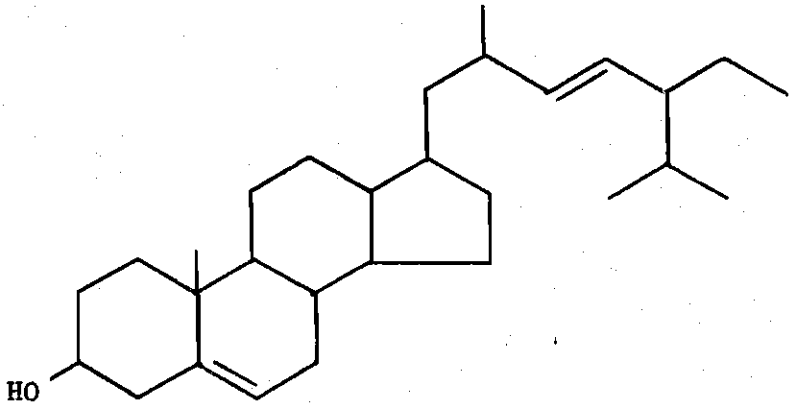


VIII

CHAPTER I

INTRODUCTION

Eupatorium rugosum, also known as White Snakeroot or richweed, is a white flowered, perennial plant found in wooded areas throughout the eastern United States. The plant is of particular interest because of its association with a disease known as "milksickness". This disease afflicted both humans and livestock in sparsely settled areas of the eastern United States throughout the nineteenth century. The literature of the period cites "milksickness" as one of the major hazards faced by the early settlers of the Appalachians and the midwest.[1]

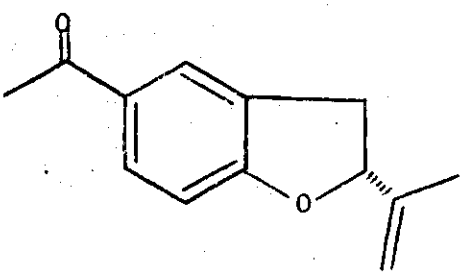
The disease is characterized in humans by a progressive increase in the severity of symptoms, such as nausea, vomiting, and weakness, leading, in severe cases, to coma and eventual death.[2]

Livestock afflicted by the disease show many of the same symptoms as humans; but also exhibit spasmodic muscular movements known as "trembles". The fact that outbreaks of trembles and milksickness appeared at the same time of year, i.e., late summer and early fall, and that those who abstained from milk and milk products were not affected by the disease, indicated that the disease was caused by a toxin

transmitted to humans through the milk of cows.[1]

Many theories were advanced as to the source of the toxin until screening experiments, such as those of Couch, established the fact that "trembles" in livestock was the result of ingesting white snakeroot.[3]

The first serious investigation into the nature of the toxin responsible for "milksickness" was done by Couch in the late 1920's. He reported the isolation of a substance from White Snakeroot which produced the disease when fed to sheep.[3] This substance, which he assumed to be homogeneous, he named "tremetol". No further significant work was done on the chemical nature of the toxin until the late 1950's, when Bonner and Degraw reported the isolation of tremetone (I), dehydrotremetone (II) and hydroxytremetone (III) from "tremetol" obtained from the plant by a modification of Couch's procedure.[4] These compounds, together with desmethylencecalin (IV)[5] are the only ones reported in the literature as having been isolated from Eupatorium rugosum.



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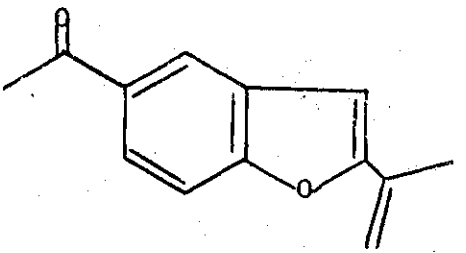

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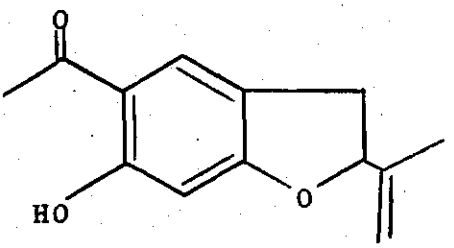

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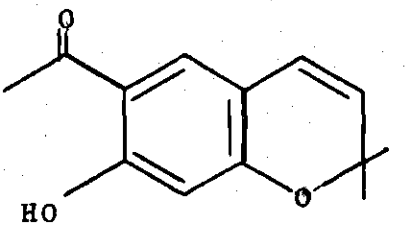
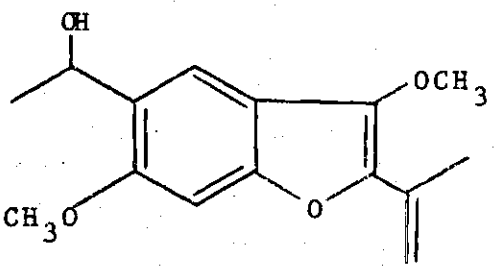


IV

Isocoma wrightii (rayless goldenrod), a plant indigenous to the southwestern United States, has also been shown to cause "milksickness". "Tremetol" obtained from rayless goldenrod by a modification of Couch's procedure has been shown to induce "milksickness" in sheep.[6] This tremetol has in turn been found to contain tremetone (I) and dehydrotremetone (II), plus several novel benzofurans.[7,8.9]

The two plants, although not closely related taxonomically, both cause the disease and both produce the secondary metabolites tremetone and dehydrotremetone. Both of these compounds have been shown to be toxic to goldfish.[10] However "trembles" in higher animals has yet to be produced by the administration of any pure compound, and as of this writing, the nature of the compound or compounds responsible for "milksickness" is still in doubt.

This thesis is the report of an investigation of an extract ("red oil") of white snakeroot, obtained by a modification of the procedures used by Couch and Bonner to prepare "tremetol". The aim of this research was to isolate and identify as many compounds as possible from the extract using gas and liquid chromatography equipment unavailable to earlier researchers on the plant. While no research into the biological activities of the compounds isolated was undertaken, two apparently new secondary metabolites have been isolated and characterized. These compounds have been named rugosumol (V) and rugosumone (VI). In addition, phytol (VII), stigmosta-S-22-dien-3B-ol (VIII), and hydroxytremetone (III) were isolated and identified and a multiply unsaturated carboxylic acid (Acid A) has been isolated and partially characterized.



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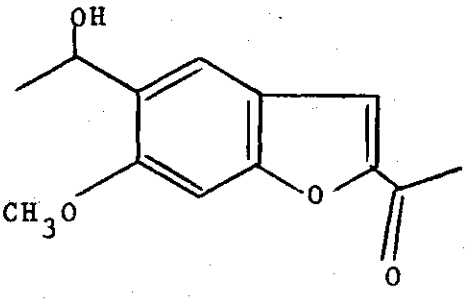

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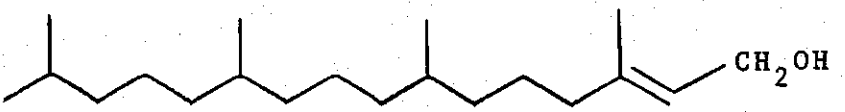

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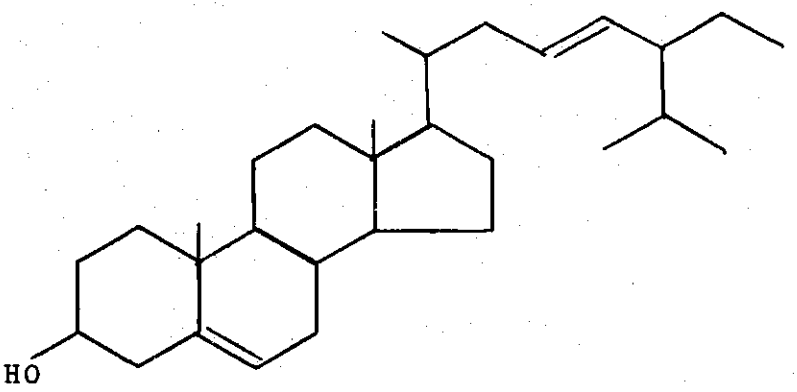

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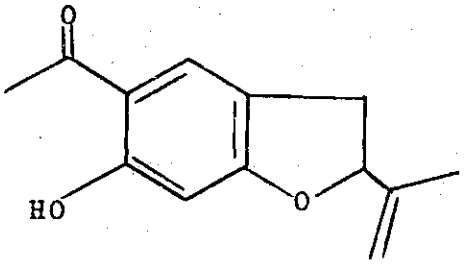


III

CHAPTER II

INSTRUMENTATION AND EQUIPMENT

Melting points were obtained on either a Thomas-Hoover capillary melting point apparatus or a Thomas-Kofler micro hot stage Model 651 and are uncorrected. Solvents were removed in vacuo on a Bucher Instruments rotary evaporator at water aspirator pressure.

All ^{1}H NMR spectra were obtained on a Varian Associates T-60A spectrometer using solutions in carbon tetrachloride or deuterochloroform with tetramethylsilane as an internal reference. The ^{13}C NMR spectrum presented in the Appendix was obtained using a JEOL PFT-100 Fourier transform spectrometer. Mass spectra and exact mass determinations were obtained using a Hitachi Perkin-Elmer Model RMU-7L mass spectrometer. Infrared spectra were done on a Perkin Elmer 237B spectrometer in carbon tetrachloride or chloroform solution. The band at 1601 cm^{-1} of polystyrene film was used as a reference. Ultraviolet spectra were done on a Perkin Elmer Model 202 spectrophotometer.

Gas chromatography was done using a F and M Model 400 gas chromatograph equipped with flame ionization detectors. The columns used are shown below:

Column Number	Liquid Phase	Support	Column Size
I	5% SE-30	80/100 Chromosorb W	4'9" x 3/8"
II	5% SE-30	80/100 Chromosorb W	5'9" x 1/4"
III	3% OV-17	100/120 Gas Chrom Q	4'9" x 3/8"

Medium pressure chromatography was done using an FMI Model RP-SY pump, with a Merck Size C silica gel 60 prepacked column, or on a Waters Associates LC-500 liquid chromatography apparatus equipped with a refractive index monitor. High pressure liquid chromatography was done on a Laboratory Data Control LC System Support I chromatography apparatus, using a prepacked Partisil M9 10/25 column by Whatman.

CHAPTER III

EXPERIMENTAL

Collection of Plant Material

Eupatorium rugosum was gathered near Unicoi, Georgia in July and August 1976. The plant was transported to the laboratory in burlap sacks and dried at room temperature in the laboratory. Directions to find the stand of *Eupatorium rugosum* from which the material was gathered were provided by Professor Sam Jones, Department of Botany of the University of Georgia.

Extraction of Plant Material (Whole Plant)

The whole plant minus the roots (604 g) was air dried and ground in a Waring blender containing 95% ethanol. The ground up leaves, suspended in ethanol, were placed in a plastic bucket and left to soak at room temperature for a period of eight weeks. The filtered ethanol solution was then evaporated *in vacuo* to leave 96 grams of dark green material. This material was dissolved in 600 ml of a 1-1 by volume methanol-water solution, 30 g potassium hydroxide were added, and the mixture was refluxed for 48 hours. The mixture was then extracted continuously with ether, and the ether evaporated *in vacuo* to leave 24.3 g of solid

material ("red oil"). This material was then subjected to analytical gas chromatography (Col I, 209°). The resulting GC trace is reproduced in Appendix I as GC Trace 1 (p. 41), with the peaks numbered for reference. GC showed the mixture to consist of three major long retention time components, and a number of smaller components with shorter retention times. Peak #7, with a longer retention time than all the others, increased in size relative to the other peaks as the concentration of the mixture injected on the column was increased. Its shape and retention time at a given temperature also varied with concentration.

Extraction of Plant Material (Leaves and Flowers)

Air dried plant material (524 g) consisting of leaves and flowers only, was ground in a Waring blender containing 95% ethanol. The ethanol containing the plant material was then placed in a glass container, and more ethanol added until the total volume was 3 liters. The material was allowed to soak in the ethanol at room temperature for three days, and then the ethanolic solution was filtered and concentrated *in vacuo* to yield 58.6 g of green material. This material was dissolved in 500 ml of 1-1 methanol water solution, 20 g of potassium hydroxide were added, and the mixture was stirred at reflux for 48 hours. The mixture was then extracted continuously with ether for 48 hours, and the ether was evaporated *in vacuo* to yield 30.1 g

of material. The material was then subjected to analytical gas chromatography (Col II, 226°). The GC trace is reproduced in Appendix I as GC trace 5 (p. 45).

Column Chromatography of Red Oil (Whole Plant)

The "red oil" obtained by extraction of the whole above ground portion of the plant (24.0 g) was prepared for chromatography by placing it on a gravity column packed with 200 grams silica gel and eluting with 2.0 ℓ ethyl acetate. The ethyl acetate was evaporated to yield 12.4 g of reddish brown material whose GC trace was identical to that of the material placed on the gravity column.

This material was then chromatographed under medium pressure using a Merck Size C silica gel 60 prepacked column. The "red oil" (3.22 g) was dissolved in 6 ml of hexane and injected onto the column. The following elution scheme was used:

Fraction #	Solvent	Volume
1-36	Hexane	.576 ℓ
37-98	Benzene	.922 ℓ
99-180	10-1 Benzene-Ethyl Acetate	1.312 ℓ
181-200	1-1 Benzene-Ethyl Acetate	.320 ℓ
201-210	Ethyl Acetate	.200 ℓ

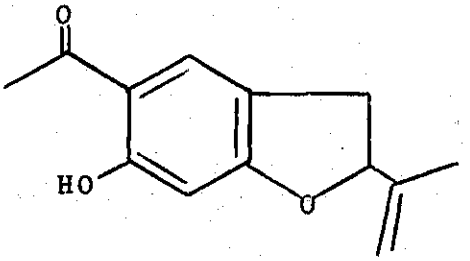
A total of 216 16 ml fractions were collected. Those fractions which appeared homogeneous by GC were combined and the solvent removed *in vacuo*. All other fractions were not utilized. The fractions combined and their weights are shown below.

Fraction #	Weight
85-89	.032 g
90-94	.093 g
106-113	.365 g
114-118	.107 g
129-131	.191 g
134-143	.150 g
144-148	.047 g
160-166	.092 g
Total	1.08 g

Isolation of Hydroxytremetone

Fractions 90-94, eluted in benzene, showed one peak on GC, R_t = 5.0 min (Col I, 209°). The fractions were combined and the solvent removed *in vacuo* to yield 93 mg of orange crystalline material. The compound had the same GC retention time as peak #4 of the original mixture, and the GC trace is reproduced in Appendix I as GC Trace 2 (p. 42). The crystalline material was identified as hydroxytremetone (III) on the

basis of the following physical and spectral data and comparison with that reported in the literature.[4,5]



III

GC: R_t = 5.0 min (Col I, 209°), (Appendix I, GC Trace 2 p. 42)

MP: 63-65° (reported m.p. 71°)

IR: $\nu^{max}_{CCl_4}$ 2924, 2860, 1642, 1480, 1330, 1255, 1132, 910 cm^{-1}

^{1}HNMR: (60 MHz, δ, $CDCl_3$): 1.75 (3H,s), 2.53 (3H,s), 2.95 (1H,m), 3.29 (1H, m), 4.93 (1H, bs), 5.07 (1H,bs), 5.26 (1H,dd,J = 9 Hz), 6.32 (1H,s), 7.48 (1H,s), 12.98 (1H,s)

MS: M^+ = 218 (8%), 175 (10%), 43 (100%)

UV: λ_{max} ($CHCl_3$) 247 (ε = 7,800), 282 (ε 10,100), 328 (ε = 7,800).

Isolation of Acid A

The gas chromatography traces of fractions 106-118 exhibited a broad peak whose retention time, shape and size relative to other components of the mixture varied, depending on the concentration of the solution injected on GC. Fractions 114-118 were the least contaminated by components with shorter retention times, and these fractions were combined and the solvent removed *in vacuo* to yield 107 mg of a waxy, white solid.

This material was further purified by HPLC. A total of 60 mg of material was injected on the HPLC equipment using a solvent system of 3-1 hexane-ethyl acetate and a Whatman partisil M9 10/25 column. A large, broad peak on the refractive index monitor (R_t=16.0 min, flow rate 80 ml/hr.) was collected and a total of 27 mg were recovered after evaporation of the solvent *in vacuo*. The material collected from HPLC had the following spectral properties:

IR: $\nu_{max}^{CCl_4}$ (cm^{-1}) 3600-2400, 2955,2920, 1701, 1410.
(Appendix II, Figure 1, p. 47).

NMR: (60 MHz, δ, $CDCl_3$) see Appendix II, Figure 2, p. 48 .

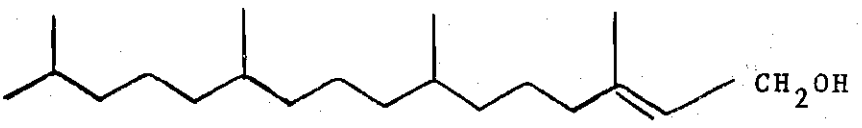
MS: 256 (8%), 43 (100%)

Isolation of Phytol

Fractions 129-131, eluted in 10-1 benzene-ethyl acetate, showed a single peak on GC, R_t = 12.2 min (Col I, 209°). These fractions were combined and the solvent removed *in vacuo*

to yield 191 mg of a yellow liquid. Although GC indicated high purity, the ^{1}H NMR spectrum of fractions 129-131 indicated possible contamination by Acid A and the material was further purified on HPLC equipment, using a Whatman partisil M9 10/25 column and solvent system of 3-1 hexane-ethyl acetate. A total of 70 mg were injected on HPLC, and a sharp peak on the refractive index monitor (R_t = 14.0 min, flow rate 80 ml/hr) was collected.

Evaporation of the solvent <u>in vacuo</u> yielded 23 mg of a yellow liquid. The material was identified as phytol (VIII) on the basis of the spectral data below, and comparison to spectra reported in the literature.[17,18,19] The material showed a single peak on GC, R_t = 12.2 min (Col I, 209°), corresponding to peak #6 of the "red oil" (Appendix I, GC Trace 1, p. 41). The GC trace of fractions 129-131 after purification by HPLC is reproduced in Appendix I as GC Trace 3. (p. 43).



VIII

GC: R_t = 12.2 min (Col. I, 209°) (Appendix I, GC Trace 3, p. 43.

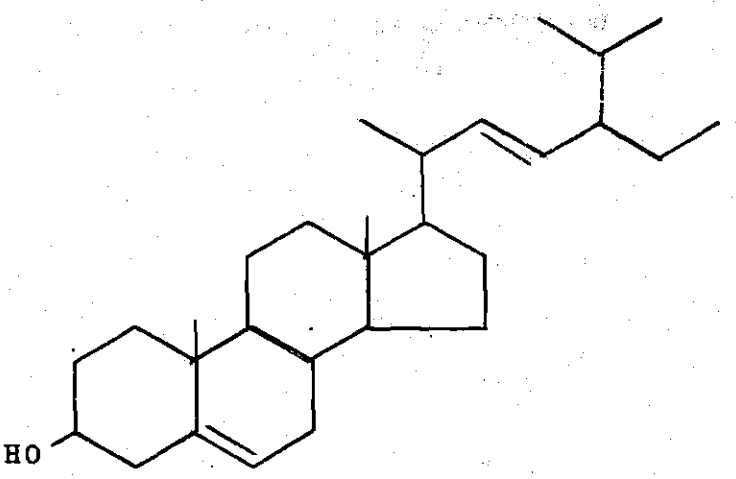
IR: ν $CHCl_3$ max (cm^{-1}) 3400, 2935, 1650, 1460, 1380, 980

^1H NMR (60 MHz, δ, $CDCl_3$): 0.83 (6H, d, J = 4 Hz), 0.84 (6H,s), 1.30 (18H, bs), 1.65 (3H,s), 2.00 (4H,m), 4.19 (2H,bd), 5.43 (1H,m)

MS = M^+ = 296 (1%), 71 (100%)

Isolation of Stigmasta-5,22-dien-3B-ol

Fractions 144-148, eluted in 10-1 benzene-ethyl acetate yielded 26 mg of white crystalline material after evaporation of the solvent in vacuo. The crystals (m.p. 160-163°C) were submitted for mass spectroscopy, but the resulting mass spectrum indicated that they were a mixture of compounds with M^+ 414 and 412 respectively. Further purification by HPLC was therefore attempted using a Whatman partisil M9 10/25 column and solvent system of 4-1 hexane-ethyl acetate. 47 mg were injected on HPLC. A sharp peak on the refractive index monitor (R_t = 33 min, flow rate 64 ml/hr) was collected. Evaporation of the solvent yielded 17 mg of white crystals, m.p. 144-147°C. Although the mass spectrum indicated that the compound with M^+ 414 was still present, the major component was identified as stigmasta-5,22-dien-3B-ol (VIII), on the basis of the following physical and spectra data, and comparison with an authentic sample.[20] The ^1H NMR and mass spectrum of the material collected from HPLC are presented in Appendix II as Figures 3 and 4. (pp. 48, 49).



VIII

GC: R_t = 7.2 min (Col. III, 274°)

MP: 144-147°C m.p. of authentic sample 161-164°C

m.m.p. with authentic sample 154-159°C

IR: ν $CHCl_3$ max (cm^{-1}) 3600, 2960, 2860, 1460, 1375, 1050, 970

^1H NMR (60 MHz, δ, $CDCl_3$): (Appendix II, Figure 3, p. 49 .)

MS: M^+ 414 (12%), 399 (4%), 396 (5%), 271 (22%), 255 (47%), 55 (100%), (Appendix II, Figure 4, p. 50 .)

Isolation of Rugosumol

Fractions 160-166, eluted in 10-1 ethyl acetate were combined and the solvent evaporated *in vacuo* to yield 92 mg of a yellow oil. Gas chromatography showed the fractions to consist of a single peak, R_t = 9.0 min., corresponding to peak #5 of the "red oil". (Appendix I, GC Trace 1, p. 41). The GC trace of fractions 160-166 is reproduced in Appendix I as GC Trace 4 (p. 44). Fractions 160-166 had the following spectral properties. The ^1H NMR, ^{13}C NMR (broad band decoupled), IR and mass spectra are reproduced in Appendix II as Figures 5, 6, 7, and 8, respectively.

GC: R_t = 9.0 min (Col. I, 209°) (Appendix I, GC Trace 4, p. 44).

BP: 120°C at 1.1 mm Hz (air bath)

IR: ν CCl_4 max (cm^{-1}) 3600, 2970, 2920, 1580, 1480, 1335, 1125, 1070. (Appendix II, Figure 5, p. 51).

^1H NMR (60 MHz, δ, $CDCl_3$): 1.51 (3H, d, J = 7 Hz), 2.10 (3H,s), 3.97 (3H,s), 4.23 (3H,s) 5.17 (2H,m), 5.78 (1H,bs), 6.57 (1H,s), 7.20 (1H,s). (Appendix II, Figure 6, p. 52)

^{13}C NMR (99.5 MHz, δ, $CDCl_3$): 19.2, 24.1, 60.3, 61.3, 65.9, 102.8, 110.5, 112.4 126.3, 132.2, 134.5, 137.2, 145.2, 145.7, 156 (Appendix II, Figure 7, p. 53).

MS: M^+ 262 (78%), 247 (51%), 244 (100%), 229 (50%), (Appendix II, Figure 8, p. 54).

Exact Mass: 262.11875 (error limit = 20 ppm)

calculated mass for $C_{15}H_{18}O_4$ = 262.12051

UV: λ_{max} (95% EtOH) 210 (ε = 20,100), 238 (ε = 11,000), 245 (ε = 10,900), 294 (ε = 19,700), 306 (sh) (ε = 15,400).

λ_{max} ($CHCl_3$) 247 (ε = 6,800), 296 (ε = 10,900), 305 (sh) (ε = 10,000)

ORD: (C = 1.14, $CHCl_3$) = $[\phi]_{589}$ = + 501.2° ($[\alpha]^{20}_{589}$ = + 191.3°)

$[\phi]_{500}$=+592.1°, $[\phi]_{500}$=+750.1°, $[\phi]_{450}$=+934.0°, $[\phi]_{400}$=+1161.9°

Rugosumol-Synthesis of Acetate

In an effort to obtain an additional amount of the material isolated as fractions 160-166 of the original chromatography, an additional amount (2.83 g) of the "red oil" from the whole plant was chromatographed. The material was dissolved in 6 ml of 3-2 hexane-ethyl acetate, injected onto a Merck Size C silica gel 60 prepacked column, and chromatographed under medium pressure. The eluting solvent system shown below was used.

Fraction	Solvent	Volume
1-33	3-2 benzene-hexane	.512 ℓ
34-59	1-1 benzene-hexane	.384 ℓ
60-79	benzene	.288 ℓ
80-156	4-1 benzene-ethyl acetate	1.216 ℓ
157-164	ethyl acetate	.128 ℓ

Fractions 120-122 contained a single peak by gas chromatography, (R.T. 9.2 min, Col. I, 209°). These fractions were combined and the solvent removed under reduced pressure to yield 107 mg of a yellow oil whole IR and ^{1}H NMR spectra were identical to those of fractions 160-166 of the original chromatography. This material (107 mg) was placed in a round bottom flask with 0.1 ml of pyridine and 1.0 ml of acetic anhydride and stirred for 24 hours at room temperature. The reaction was quenched with 4 ml distilled water and the aqueous solution was extracted twice with 5 ml portions of ether. The ether soluble portion was then washed with sodium bicarbonate, dried over magnesium sulfate, and the ether evaporated in vacuo to yield 79 mg of a yellow oil. Gas chromatography of the reaction product showed it to be 90% pure, with a longer retention time than the starting material. It exhibited the spectral properties shown below:

GC: R_t = 12.2 min (Col. I, 209°)

IR: $\nu_{max}^{CCl_4}$ (cm^{-1}) 2933, 1750, 1460, 1325, 1245, 1110, 897 (Appendix II, Figure 9, p. 55).

^{1}H NMR (60 MHz, δ, $CDCl_3$): 1.55 (3H, d, J=7 Hz), 2.08 (6H, s), 3.98 (3H, s), 4.25 (3H, s), 5.20 (1H, bs), 5.78 (1H, bs), 6.27 (1H, q, J=7 Hz), 6.58 (1H, s), 7.23 (1H, s) (Appendix II, Figure 10, p. 56).

MS: M^+ = 304 (100%), 289 (7%), 261 (6%), 245 (39%).

Exact Mass: 304.12802 (error limit 20 ppm)

Calculated mass for $C_{17}H_{20}O_5$: 304.13108

UV: λ_{max} (95% EtOH) 208 (ε = 18,100) 242 (ε = 9,100)
248 (ε = 8,800) 290 (ε = 16,300) 305 (ε = 12,900)

Column Chromatography of "Red Oil" (Leaves and Flowers)

The "red oil" (30.0 g) prepared by extraction of the leaves and flowers was placed on a gravity column filled with 200 g silica gel (230-400 mesh) and eluted with 2.0 liter ethyl acetate. The ethyl acetate was then removed *in vacuo* to yield 17.0 g of reddish brown material. This material (10.15 g) was injected onto a Waters 500 preparative liquid chromatography apparatus. The material was chromatographed using 10-1 ethyl acetate as the eluting solvent. The chromatography was monitored using a refractive index monitor. A total of 13 fractions were collected, and their weights and volumes are shown below.

Fraction	Volume	Weight
1	.060 ℓ	.323 g
2	.150 ℓ	.469 g
3	.250 ℓ	.473 g
4	.380 ℓ	2.149 g
5	.250 ℓ	.450 g
6	.300 ℓ	.422 g
7	.300 ℓ	.452 g
8	.300 ℓ	.151 g

9	1.100 ℓ		.224 g
10	.500 ℓ		.086 g
11	1.750 ℓ		.126 g
12	1.100 ℓ		1.984 g
13	1.000 ℓ		.090 g
		Total	7.399 g

All fractions were submitted to analytical gas chromatography, and the solvent removed *in vacuo*.

Isolation of Rugosumone

Fraction 11 of the chromatography of the "red oil" from the leaves and flowers appeared to contain crystalline material after evaporation of the solvent. The crystalline material did not dissolve in 5 ml anhydrous ether, and filtration produced 7 mg of solid material. The GC trace of this material indicated that it was analytically pure, and all the physical and spectral data shown below are those of the material obtained by filtration. However, that portion of fraction 11 which was soluble in ether (119 mg) was rechromatographed on HPLC equipment in an effort to obtain an additional amount of the compound. The refractive index monitor of the HPLC equipment showed three major peaks when the mixture was injected on HPLC using a solvent system of 2-1 hexane ethyl acetate. The second peak to appear (R_T = 31 min, flow rate 96 ml/hr) was collected. Evaporation

of the solvent under a stream of nitrogen yielded 4 mg or orange crystals (mp 110-120°C), with the same GC retention time as the material obtained by filtration.

GC: R_t = 9.1 min (Col. II, 226°)

MP: 178-182° (from ether)

IR: $\nu_{max}^{CHCl_3}$ (cm^{-1}) 3600, 3000, 2960, 2925, 1638, 1620, 1550, 1470, 1120, 995 (Appendix II, Figure 11, p. 57 .)

^1H NMR (60 MHz, δ, $CDCl_3$) = 1.51 (3H, d, J=7 Hz), 2.56 (3H, s), 3.95 (3H, s), 5.20 (1H, q, J=7 Hz), 7.71 (1H, s), 7.46 (1H, s), 7.06 (1H, s) (Appendix II, Figure 12, p. 58 .)

MS: M^+ 234 (12%), 219 (23%), 216 (61%), 43 (100%)

(Appendix II, Figure 13, p. 59 .)

Exact Mass: 234.073949 (error limit 100 ppm)

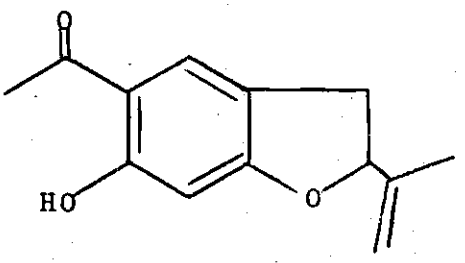
Calculated mass for $C_{13}H_{14}O_4$ = 234.089210

UV: λ_{max} (95% EtOH): 205 (ε = 6000), 215 (sh) (ε = 5100) 245 (ε = 1800), 327 (ε = 10600)

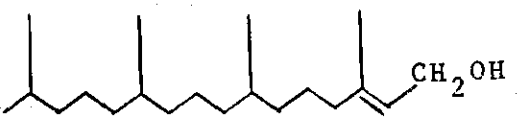
CHAPTER IV

DISCUSSION OF RESULTS

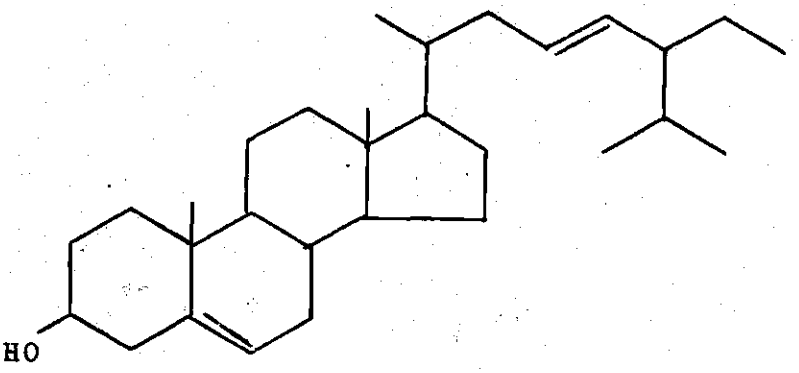
In the course of this investigation, five compounds have been isolated and identified from white snakeroot. Hydroxytremetone (III) has been found in the plant by Bonner and DeGraw[4] and also by Zalkow and Ghosal in this laboratory.[5] Stigmasta-9-22-dien-3B-ol (VIII) and phytol (VII), which occur quite commonly in nature, have never been reported before in white snakeroot. Rugosumol (V) and rugosumone (VI) have been isolated and assigned the structures shown on the basis of spectroscopic evidence presented in this chapter. A search of the literature has failed to produce a report of these compounds' ever having been found in nature.



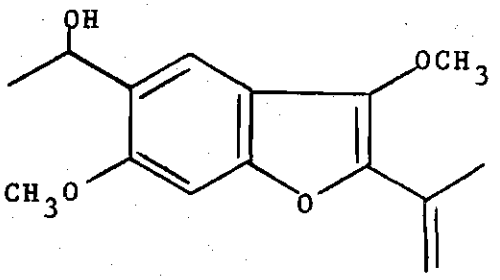
III



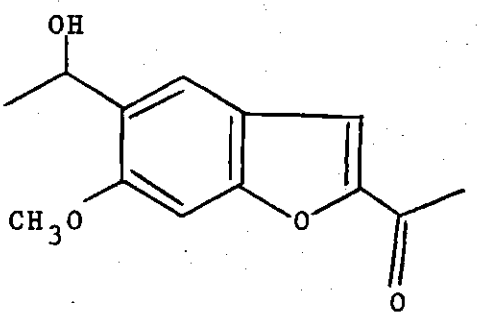
VII



VIII

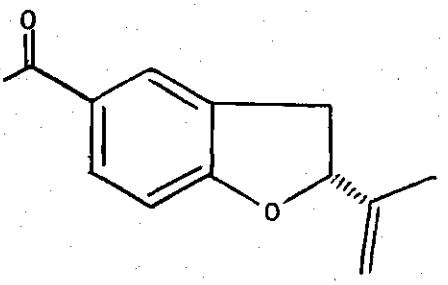


V

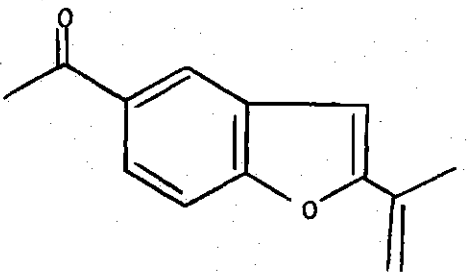


VI

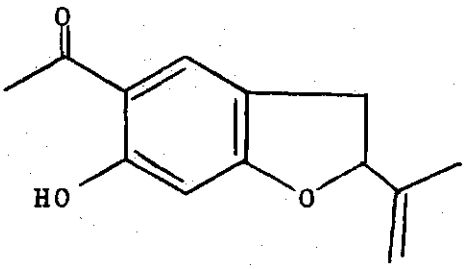
As stated in the introduction, the extraction procedure used in this study is an abbreviation of that used by Bonner and DeGraw to prepare "tremetol" from white snakeroot, from which they isolated hydroxytremetone (III), tremetone (I) and dehydrotremetone (II). Bonner's extraction procedure is shown in Chart I, (p. 26).[4]



I



II

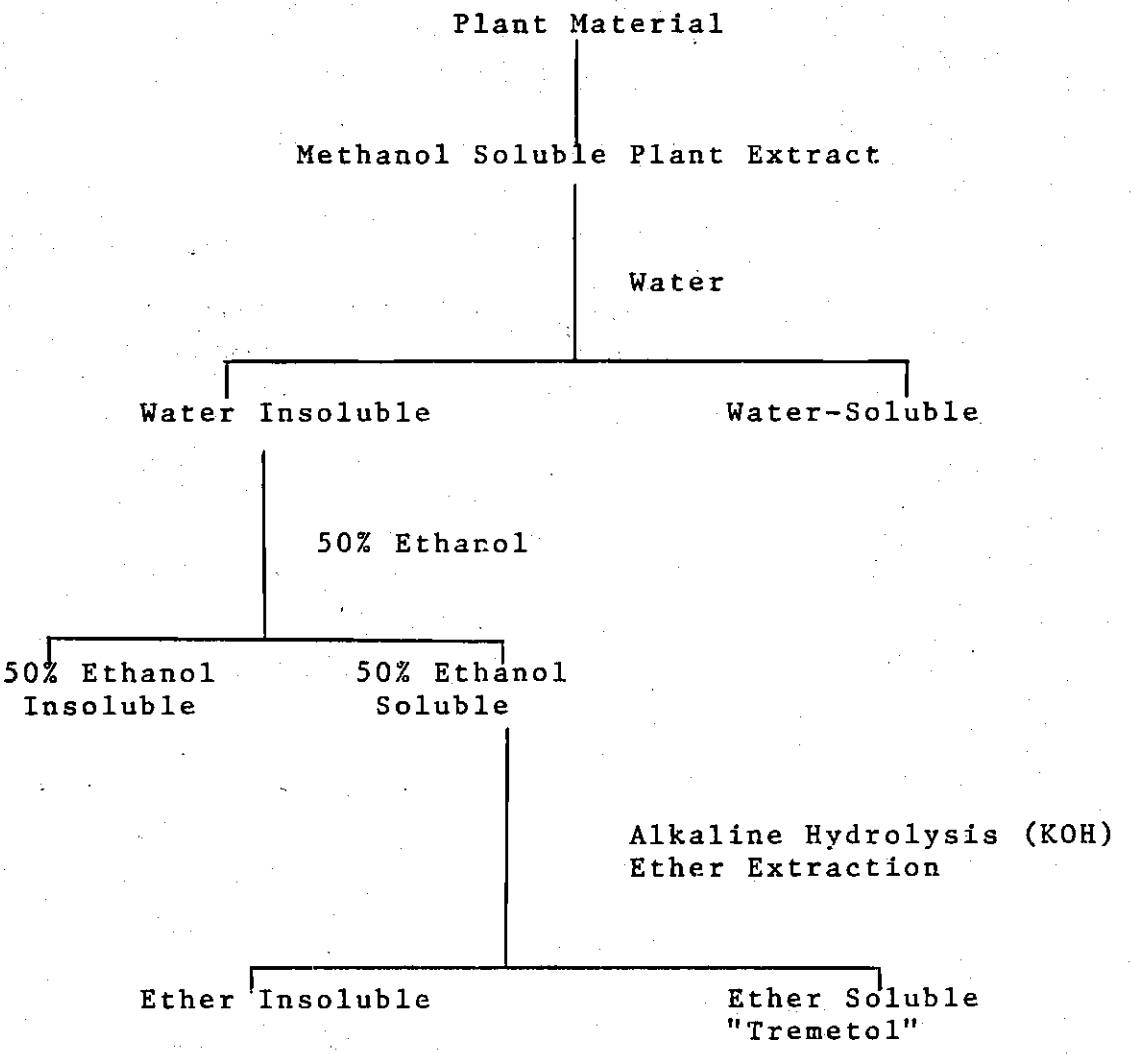


III

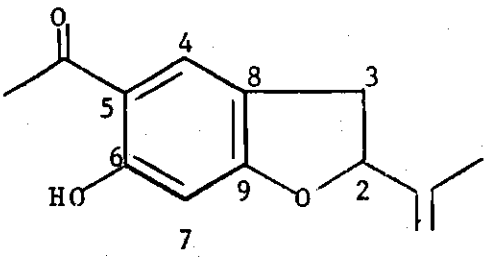
Of these three compounds, only hydroxytremetone was isolated from the "red oil". Mixed GC injections of tremetone (I) and dehydrotremetone (II) isolated from Isocoma wrightii (rayless goldenrod) with "red oil" prepared from the leaves and flowers of white snakeroot failed to detect these compounds presence. The similarity of Bonner's extraction procedure to that used in this study, and the structural similarity of tremetone and dehydrotremetone to hydroxytremetone, lead to the conclusion that tremetone and dehydrotremetone, if present in the plant, should be found in

the "red oil". Their absence may be due to phytochemical differences between the plant studied by Bonner, which was grown in Indiana, and that used in this study, from the Appalachian Mountains of Georgia.

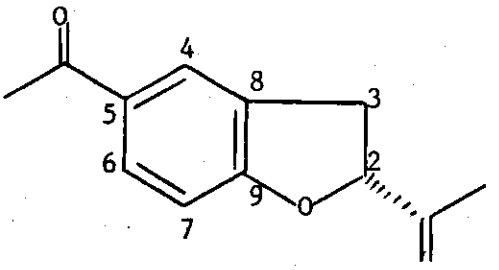
CHART 1. BONNER'S EXTRACTION PROCEDURE TO PREPARE "TREMETOL"



Hydroxytremetone was isolated by medium pressure chromatography in sufficient purity to permit its identification without further purification. Comparison of physical and spectral data with those reported by Zalkow[5] and Bonner[4] revealed no discrepancies. Hydroxytremetone has the structure III, but the configuration about the asymmetric carbon has not been proven. The only other benzofuran from white snakeroot with an asymmetric carbon at C-2 is tremetone (I) which has been shown to have the R configuration at C-2 as shown below.[11]



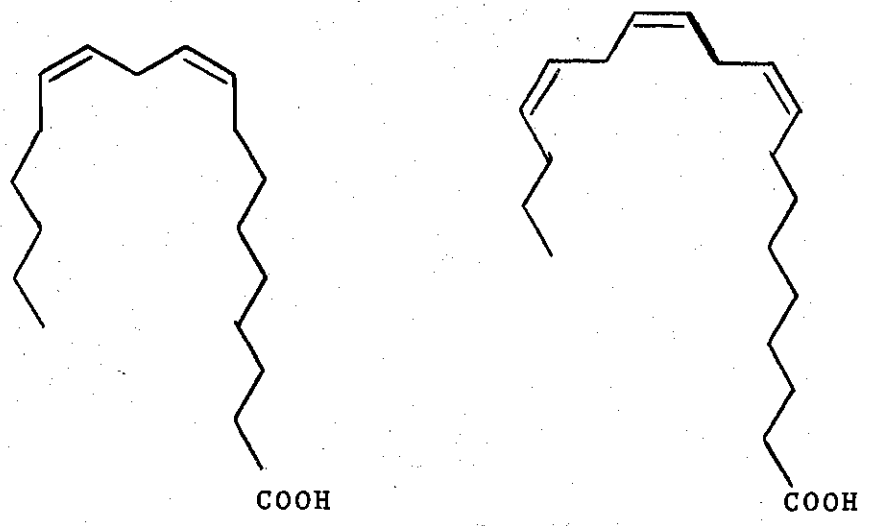
III



I

The compound referred to as Acid A had a broad band in its IR spectrum (Appendix II, Figure 1, p. 47) at 3600-2400 cm^{-1} and a strong carbonyl band at 1705 cm^{-1} which suggested the presence of a carboxylic acid moiety. The NMR spectrum (Appendix II, Figure 2, p. 48) is similar to those

of linoleic acid (IX)[12] and linolenic acid (X)[13], both of which occur commonly in nature.

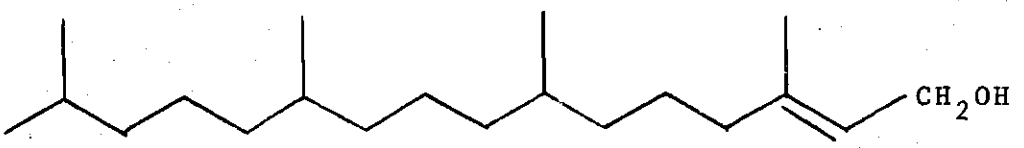


IX X

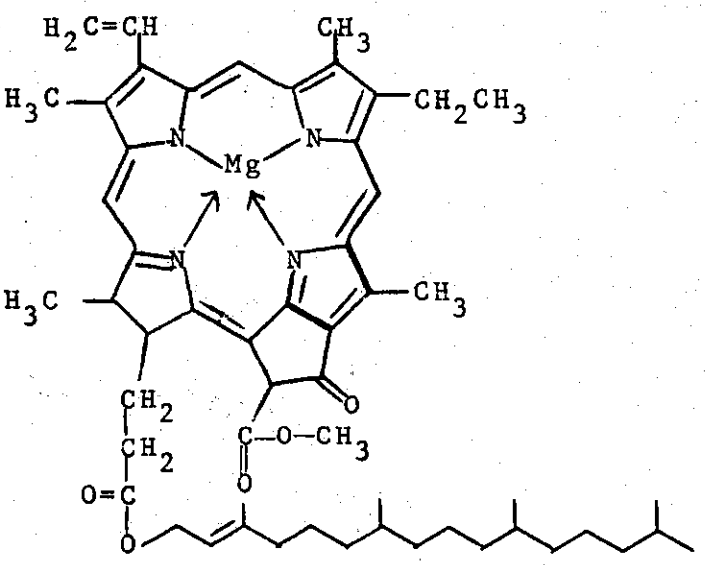
The ratio of the integration of the ^{1}H NMR signals of the olefinic protons at δ5.37 and the methylene protons between double bonds at δ2.80 should distinguish between these two compounds. In the NMR spectrum of Acid A, this ratio was 16:10, which approximates the 3:2 ratio of linolenic acid. However, the mass spectrum of Acid A showed an apparent molecular ion of m/e 256 and a base peak at m/e 43, which in no way corresponds to the published mass spectra of linoleic acid (IX)[14] or linolenic acid (X).[15] Because of its apparent tendency to deteriorate during storage, further efforts to determine the structure of Acid A were not pursued.

Gas chromatography showed phytol to be the major component of the extract, but it proved difficult to isolate in pure form. Fractions 129-131 from medium pressure chromatography, although pure by GC, contained signals in their ^{1}H NMR spectrum at δ5.37 and δ2.80 which indicated possible contamination by Acid A. The spectral data given in the experimental section and the gas chromatography trace in Appendix I (GC Trace 3, p. 43) all pertain to 23 mg of material collected by HPLC. The ^{1}H NMR, IR, and mass spectrum of this material was identical to that published for phytol.[16,17,18]

Phytol has the structure (VII). It is rarely found free in nature, occuring mainly in ester form with chlorophyll (XI).[19] It occurs in the "red oil" probably as an artifact of the base hydrolysis used to prepare the extract.

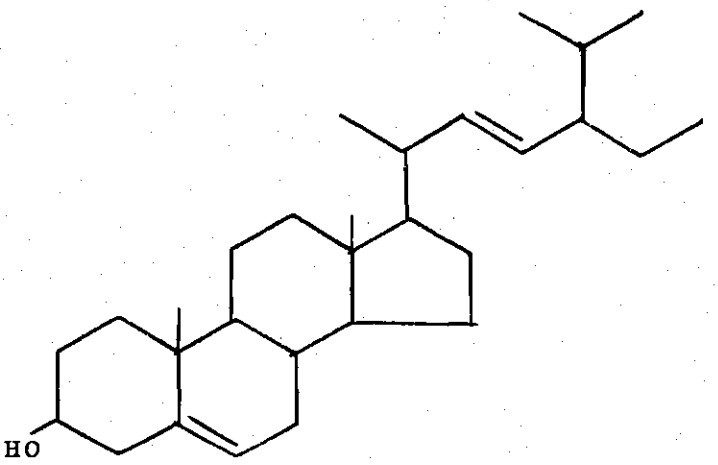


VII



XI

Stigmasta-5,22-dien-3B-ol (VIII) is one of the most common of naturally occurring phytosterols. It was not isolated in pure form even by HPLC, but was contaminated with a compound of molecular weight 414.

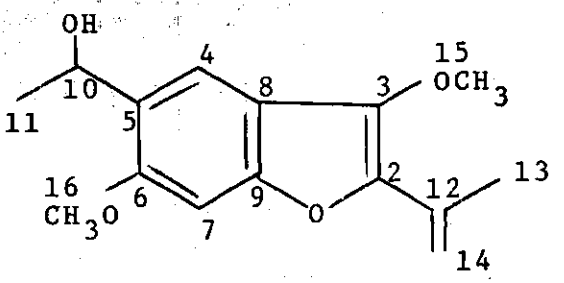


(VIII)

The mass spectrum of the material obtained from HPLC purification of fraction 144-148 of the "red oil" was compared with that of an authentic sample of (VIII).[20] All of the peaks in the high mass region of (VIII) were also present in the mass spectrum of the material obtained from the "red oil" (Appendix II, Figure 3, p. 49). These include peaks at m/e 412 (23%), 395 (5%), 369 (6%), 351 (7%), 271 (22%), and 255 (47%). The identity of the material from the "red oil" as VIII was confirmed by mixed GC injections with an authentic sample. The major component of the material from the "red oil" had the same retention time (R_t = 7.2 min, Col. III, 274°) as the authentic sample of VIII.

Structure of Rugosumol

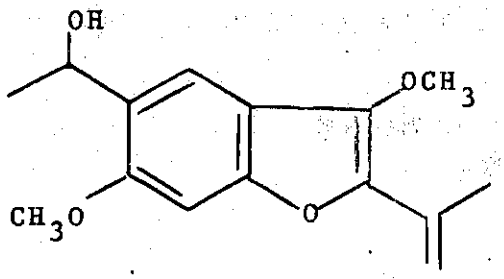
Gas chromatography showed rugosumol to be one of the three major components of the "red oil" from the whole plant corresponding to peak #5 of the mixture. (Appendix I, GC Trace I, p. 41). It was isolated in high purity by medium pressure chromatography. The structure of rugosumol, with the exception of the configuration about the asymmetric center at C-10 has been assigned as (V) on the basis of the following spectroscopic evidence.



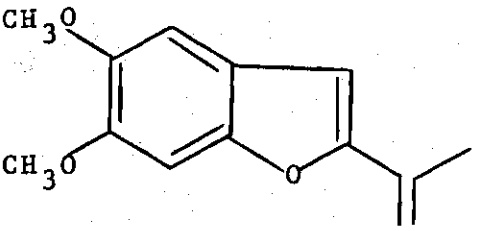
V

(1) Exact mass determination indicated a molecular formula of $C_{15}H_{18}O_4$.

(2) The UV spectrum (MeOH) was similar to that of (XII),[21] the only other naturally occurring benzofuran with a similar conjugated system for which UV data has been published.



V



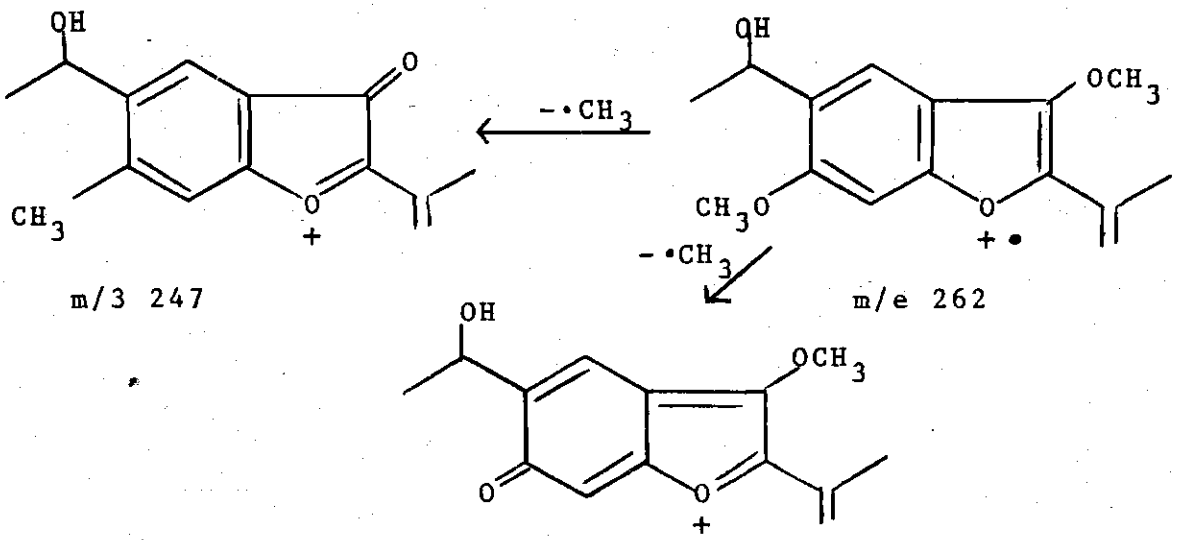
XII

λ_{max} (EtOH)		λ_{max} (MeOH)	ϵ
210	20,100	215	15,100
238	11,000	278	10,500
245	10,900	287	10,000
294	19,700	314	16,200
306 (sh)	15,400	324 (sh)	13,500

(3) The 60 MHz ^{1}H NMR spectrum (Appendix II, Figure 6, p. 52) indicated the presence of two aromatic protons as sharp singlets at δ6.57 and δ7.20, two methoxyl groups as sharp singlets at δ3.97 and δ4.23, a methyl adjacent to one proton as a doublet centered at δ1.51 (J = 7 Hz) and a methyl on a double bond as a singlet at δ2.10. One of the olefinic protons was visible as a broad singlet at δ5.78, the other being obscured by the signal of the methine proton at C-10.

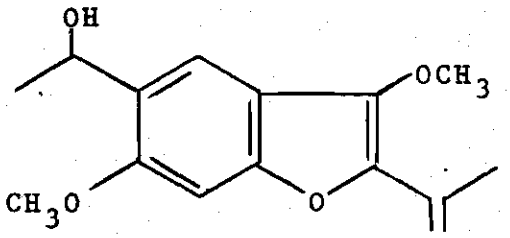
(4) IR showed the presence of a hydroxyl group (3600 cm^{-1}) and a terminal double bond (890 cm^{-1}) (Appendix II, Figure 5, p. 51).

(5) The mass spectrum (Appendix II, Figure 8, p. 54) showed an abundant molecular ion at m/e 262 (78%) which is characteristic of the mass spectra of benzofurans. The large peak at m/e 244 (100%) due to loss of H_2O corroborated the IR spectrum in suggesting the presence of a hydroxyl group. The peak at m/e 247 due to loss of methyl may be interpreted as shown below.

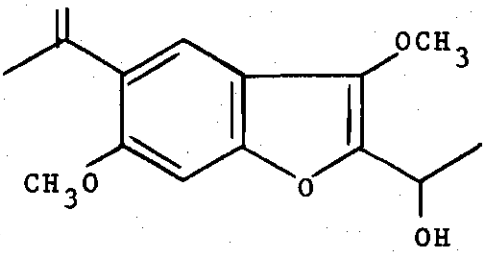


The spectral data indicate a benzofuran ring substituted at four positions. The signals of the two aromatic protons appear as sharp singlets in the ^1H NMR spectrum and must therefore be oriented para to one another on the aromatic ring. Three possible structures which would conform to the

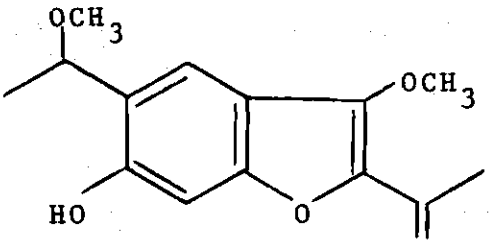
spectral data are shown below.



V

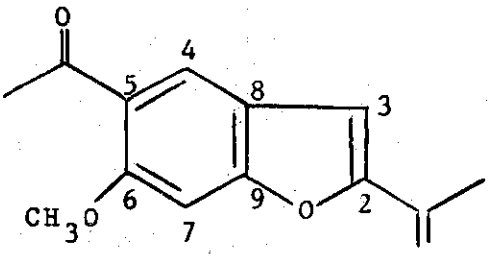


XIII



XIV

Structure XIII can be eliminated as a possibility primarily on the basis of biosynthetic evidence. A study of the biosynthesis of dehydrotremetone (II) in Eupatorium rugosum using isotopic labeling techniques has shown that the acetophenone portion of the molecule was derived from a polyacetate precursor while the furan ring was derived from an isoprenoid precursor.[22]



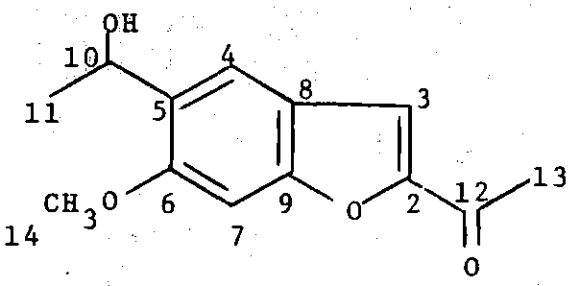
II

Such a pathway could not lead to a benzofuran with a two carbon substituent at C-2, such as XIII. Of 54 naturally occurring benzofurans reported in the literature by 1976[23] none have a three carbon substituent at C-5, as does structure XIII.

Structure XIV was eliminated as a possibility on the basis of the ^1H NMR spectrum of the acetate formed by treating rugosumol with acetic anhydride in pyridine. Acetylation of the hydroxyl group of structure V should result in a downfield shift of the signal of the proton at C-10 of about 1 ppm. Acetylation of the phenolic hydroxyl group of structure XIV should result in little or no change in the position of the signal of the C-10 proton. In the ^1H NMR spectrum of the acetate of Rugosumol (Appendix II, Figure 10, p. 56) the signal of the C-10 proton is clearly seen as a quartet (J = 7 Hz) centered at δ6.27, a downfield shift of about 1 ppm as compared to rugosumol. The doublet corresponding to the C-11 methyl group also underwent a slight downfield shift in forming the acetate (δ1.51 to δ1.55), but all of the other signals remained unchanged.

Structure of Rugosumone

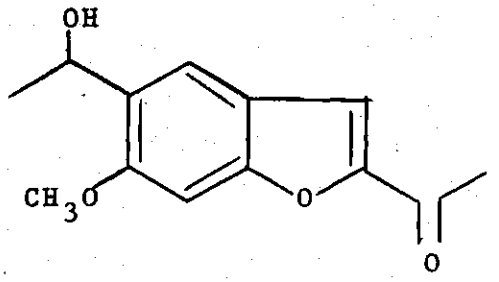
Rugosumone (VI) was isolated as a crystalline solid from "red oil" prepared from the leaves and flowers of white snakeroot. Its GC retention time (9.10 min, Col. II, 226°) was nearly identical with that of Rugosumol, and no estimation of its relative abundance in the mixture could be made from GC. A total of 12 mg were isolated from chromatography of 10.15 g of "red oil". Rugosumone has been assigned the structure VI on the basis of the following spectroscopic evidence.



VI

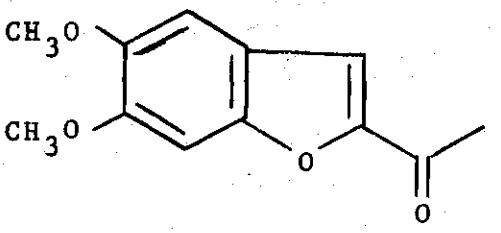
(1) Exact mass determination indicated a molecular formula of $C_{13}H_{14}O_4$.

(2) The UV spectrum of rugosumone was similar to that of the synthetically prepared (XV) which has nearly the same conjugated system.[22]



VI

λ_{max} (MeOH)	ε
205	6,000
215	5,100
245	1,800
327	10,600



XV

λ_{max} (MeOH)	ε
222	15,100
263	6,400
298 (sh)	14,100
339	23,400

(3) The 60 MHz ^{1}H NMR spectrum (Appendix II, Figure 12, p. 58) indicated three aromatic protons as sharp singlets at δ7.71, δ7.46, and δ7.06, a methoxyl group as a sharp singlet at δ3.95, a methyl adjacent to one proton as a doublet centered at δ1.51 (J = 6 Hz), a methine proton adjacent to a methyl group as a quartet centered at δ5.20 (J = 6), and an acetyl methyl as a singlet at δ2.57.

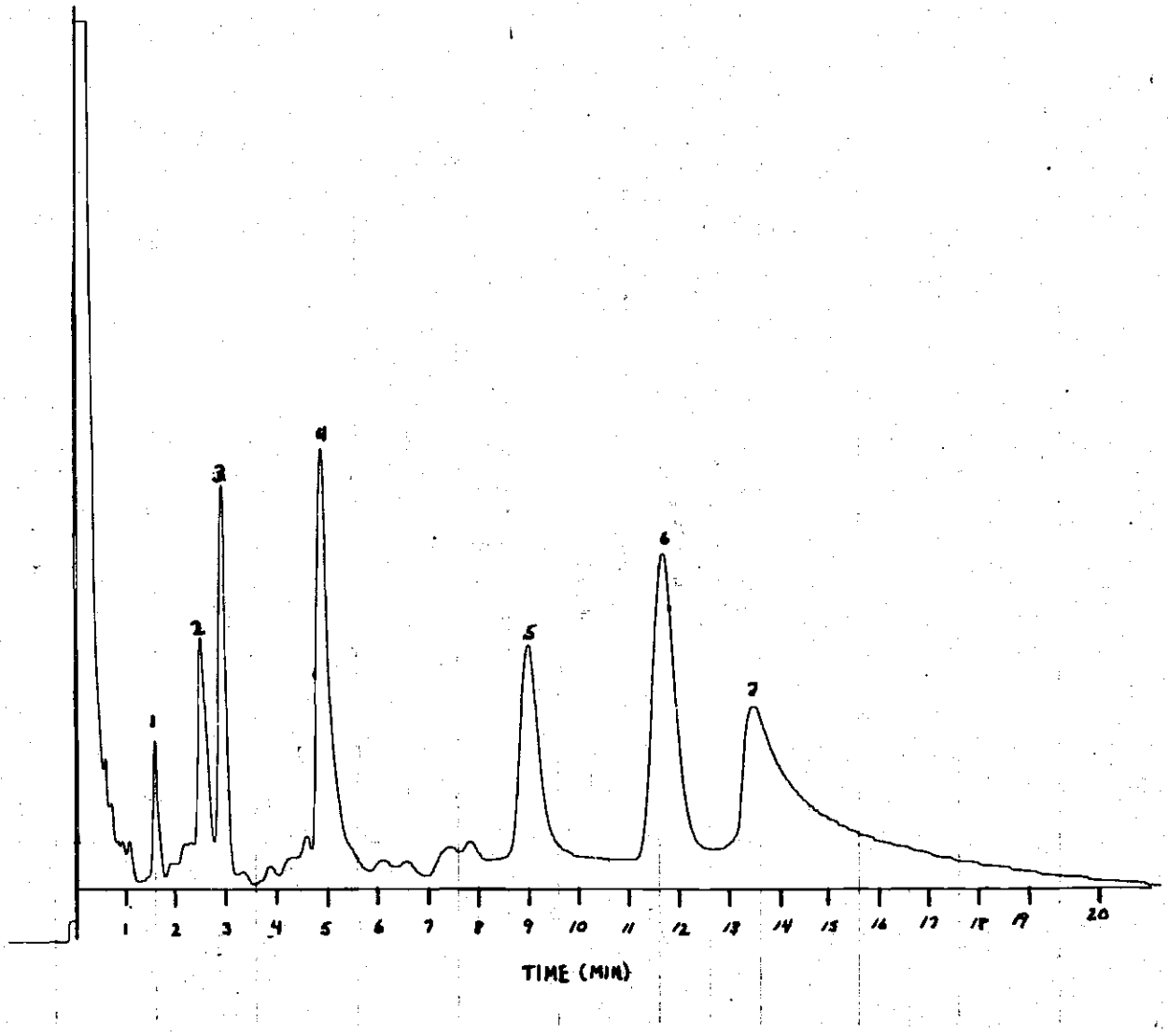
(4) The IR spectrum showed the presence of a hydroxyl group (3600 cm^{-1}) and a conjugated ketone (1638 cm^{-1}). (Appendix II, Figure 13, p. 59).

(5) The mass spectrum (Appendix II, Figure 13, p. 59) showed a molecular ion at m/e 234 (12%). it was similar to that of rugosumol (Appendix II, Figure 8, p. 54) in that it contained an abundant peak corresponding to loss of H_2O at m/e 216 (61%), a peak corresponding to loss of a methyl radical at m/e 219 (23%) and a peak corresponding to loss of H_2O and methyl at m/e 201 (31%).

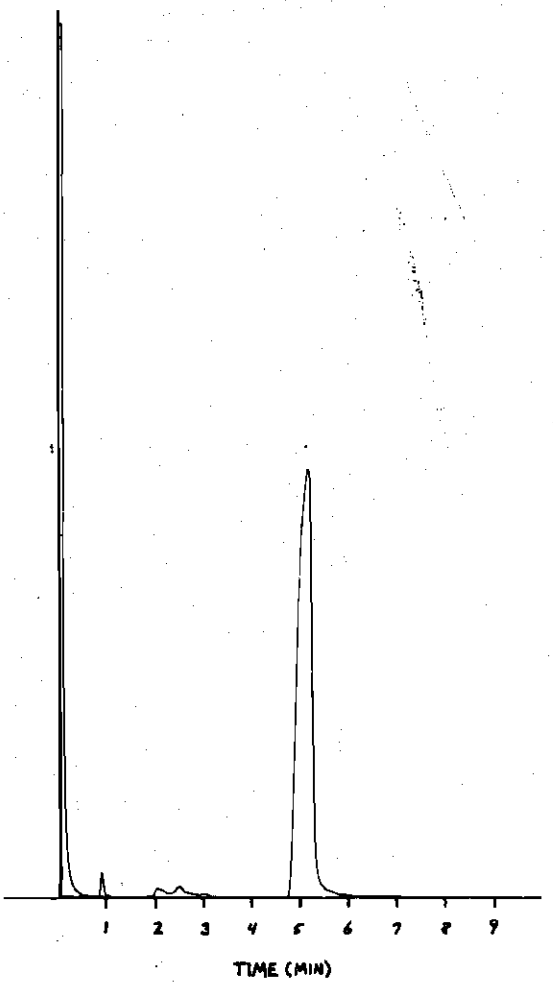
The above spectral data indicate a benzofuran ring substituted at three positions. Placement of the substituents at C-2, C-5, and C-6 of the benzofuran ring is the only possible arrangement in which the ^1H NMR signals of the aromatic protons would appear as sharp singlets. Placement of the acetyl group at C-2 and the hydroxyethyl group at C-5 of the benzofuran ring is based upon a biosynthetic argument similar to that given for rugosumol. The methoxyl group must therefore be located at C-6 of the benzofuran ring.

APPENDIX I

GAS CHROMATOGRAPHY TRACES

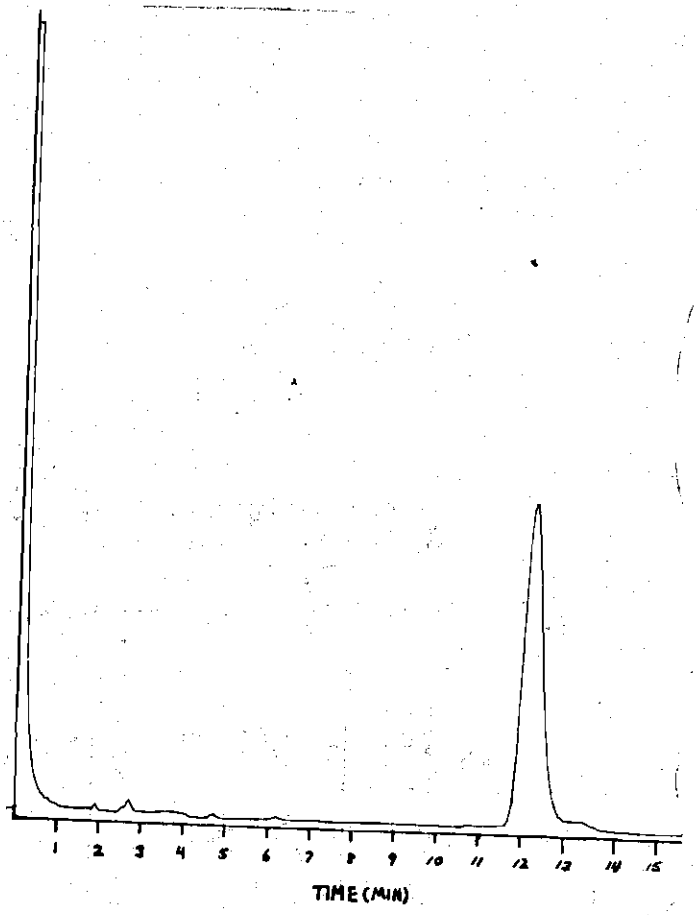


GC TRACE I - "RED OIL" FROM WHOLE PLANT (COL. I, 209°)

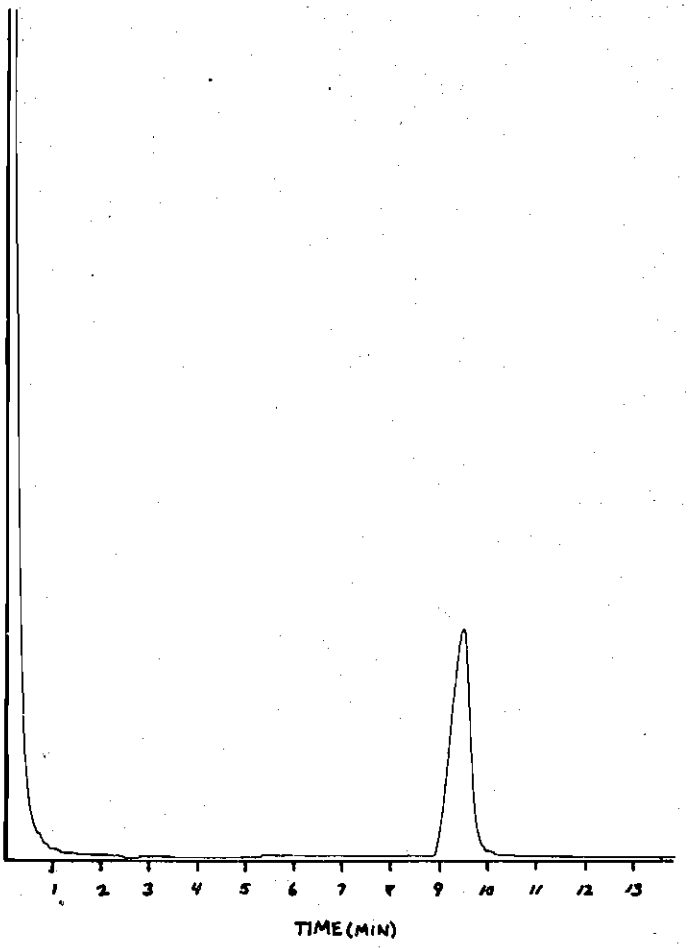


GC TRACE 2 - FRACTION 90-94 (HYDROXYTREMETONE)

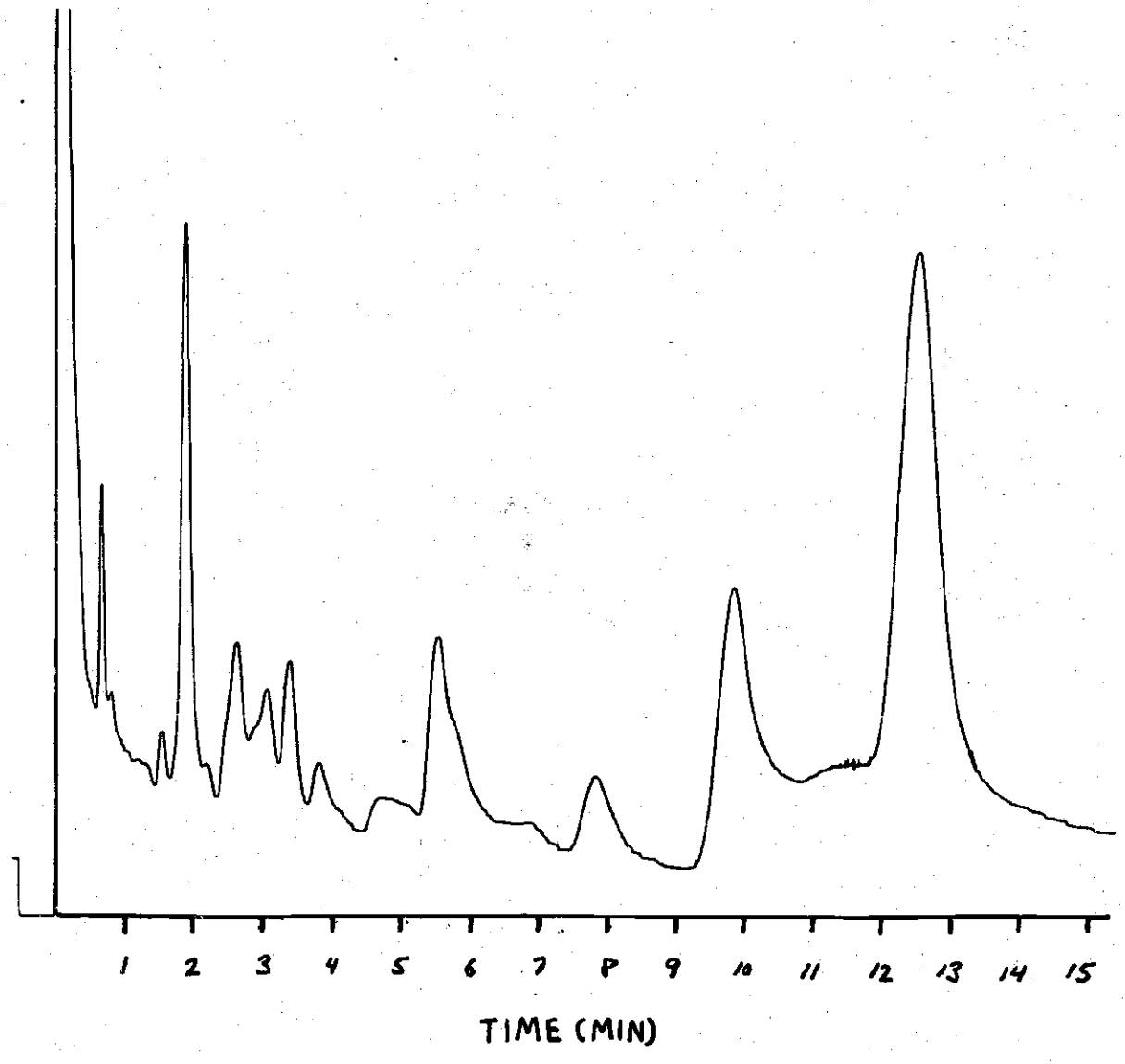
(COL. I, 209°)



GC TRACE 3 - FRACTION 129-131 AFTER PURIFICATION BY HPLC (PHYTOL) (COL. I, 209°)



GC TRACE 4 - FRACTION 160-166 (RUGOSUMOL) (COL. I, 209°)



GC TRACE 5 - "RED OIL" FROM LEAVES AND FLOWERS (COL II, 226°C)

APPENDIX II

NMR, IR AND MASS SPECTRA

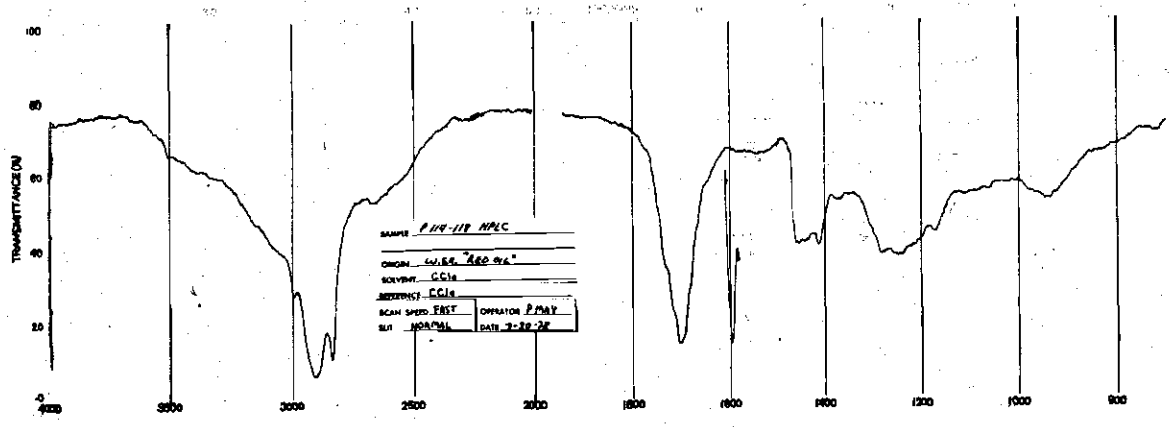


FIGURE 1. IR SPECTRUM (CCl_4) OF FRACTION 114-118 AFTER PURIFICATION BY HPLC (ACID A)

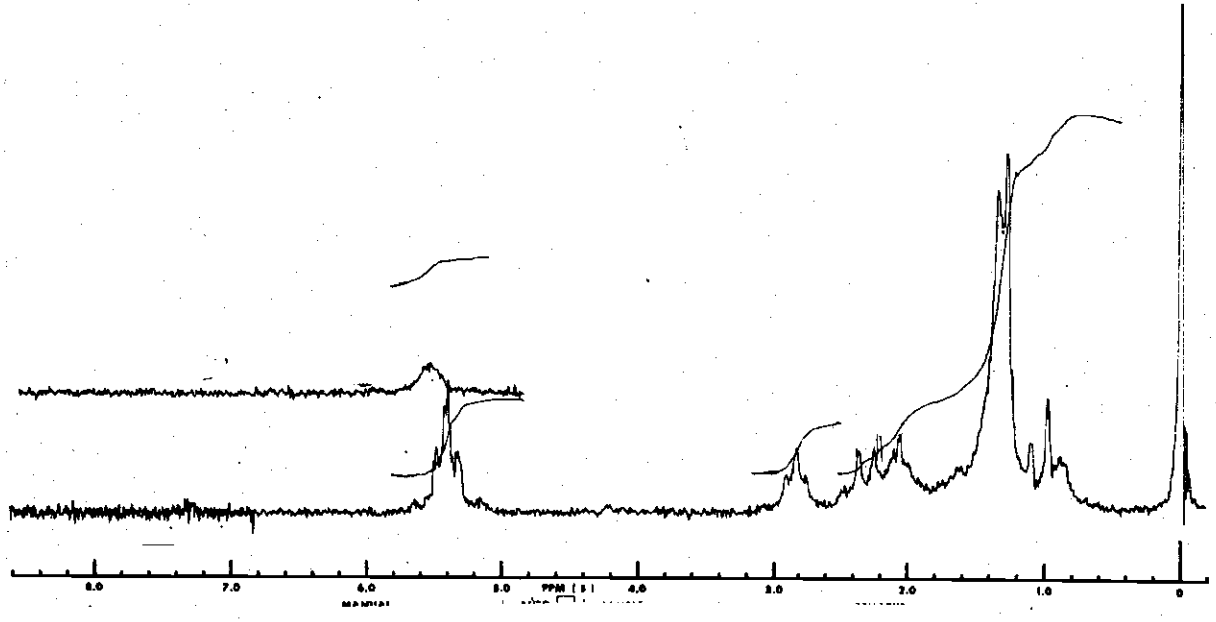


FIGURE 2. ^{1}H NMR SPECTRUM (60 MHz, $CDCl_3$) OF FRACTION 114-118 AFTER PURIFICATION BY HPLC (ACID A) (UPPER TRACE OFFSET 200 Hz)

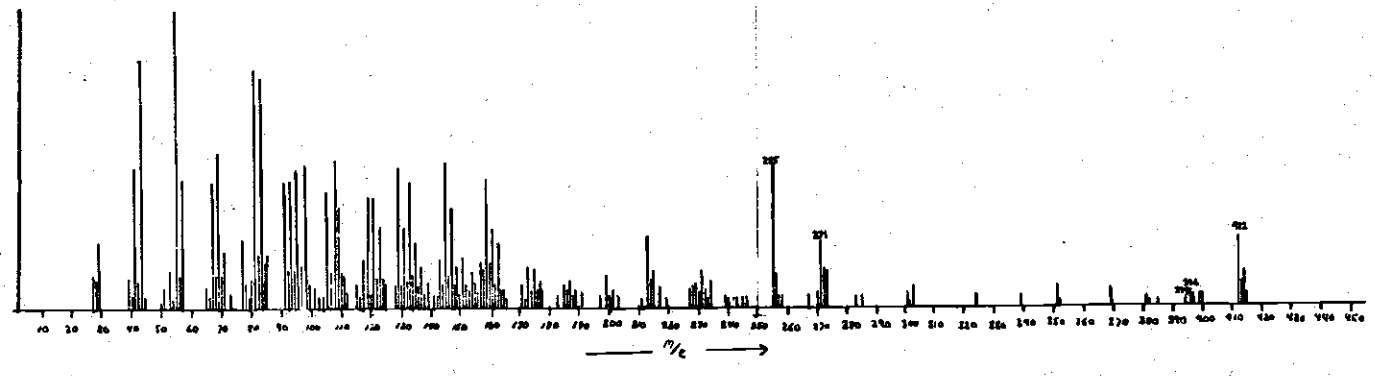


FIGURE 3. MASS SPECTRUM OF FRACTION 114-118 AFTER PURIFICATION BY HPLC (STIGMASTA-5,22-DIEN-3B-OL)

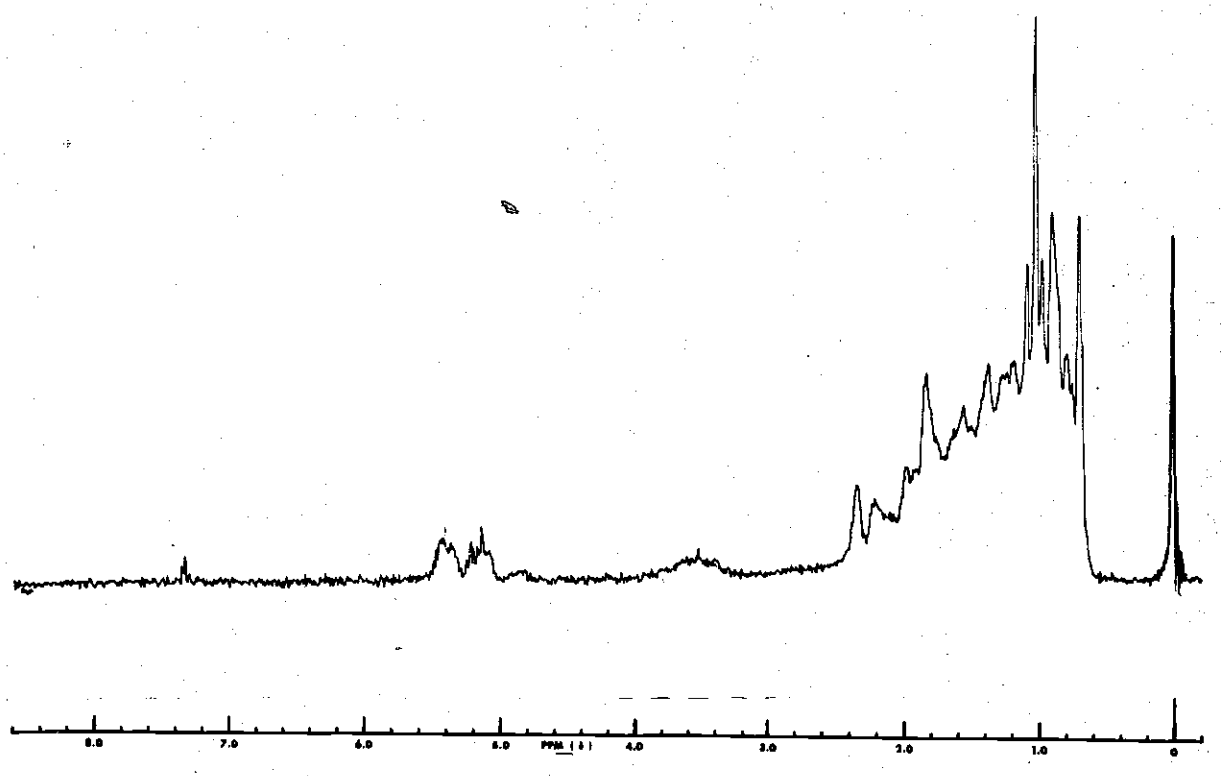


FIGURE 4. ^{1}H NMR SPECTRUM OF FRACTIONS 144-148 AFTER PURIFICATION BY HPLC (STIGMASTA 9-22-DIEN-3B-OL)

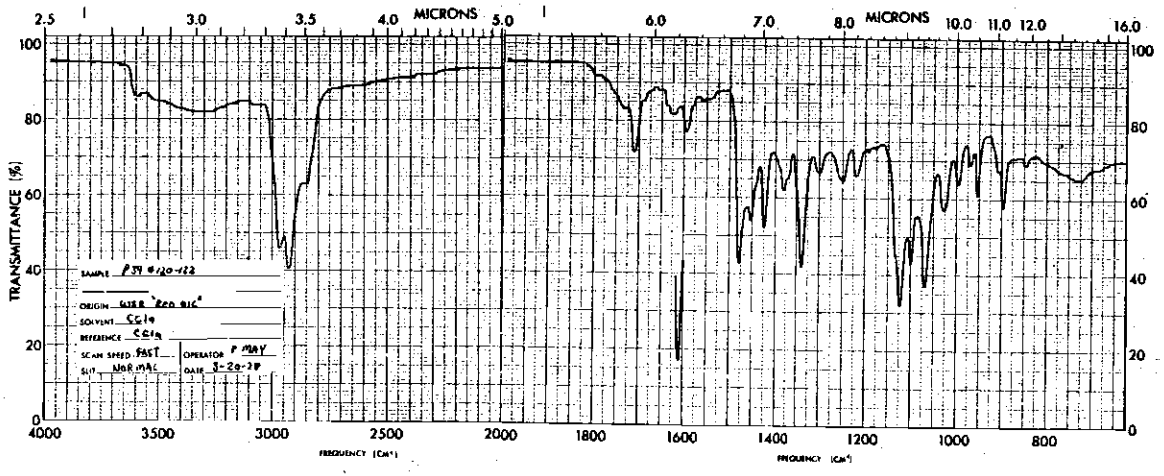


FIGURE 5. IR (CCl_4) OF RUGOSUMOL

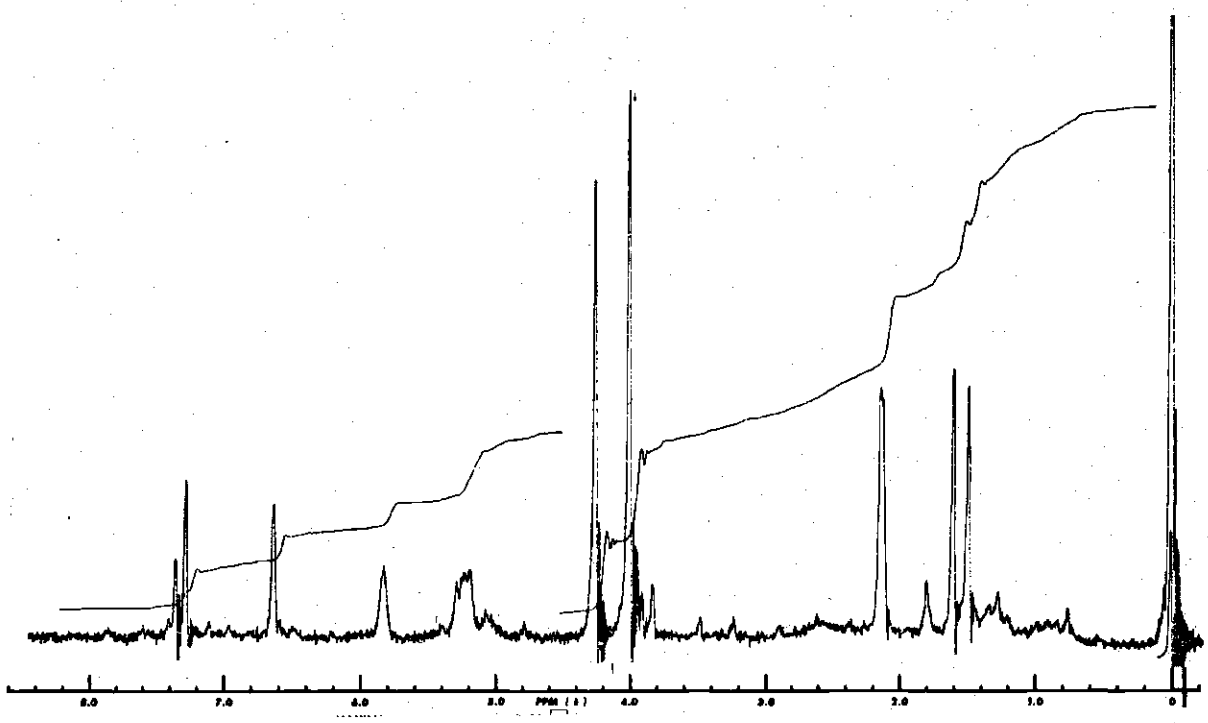


FIGURE 6. ^{1}H NMR SPECTRUM (60 MHz, $CDCl_3$) OF RUGOSUMOL

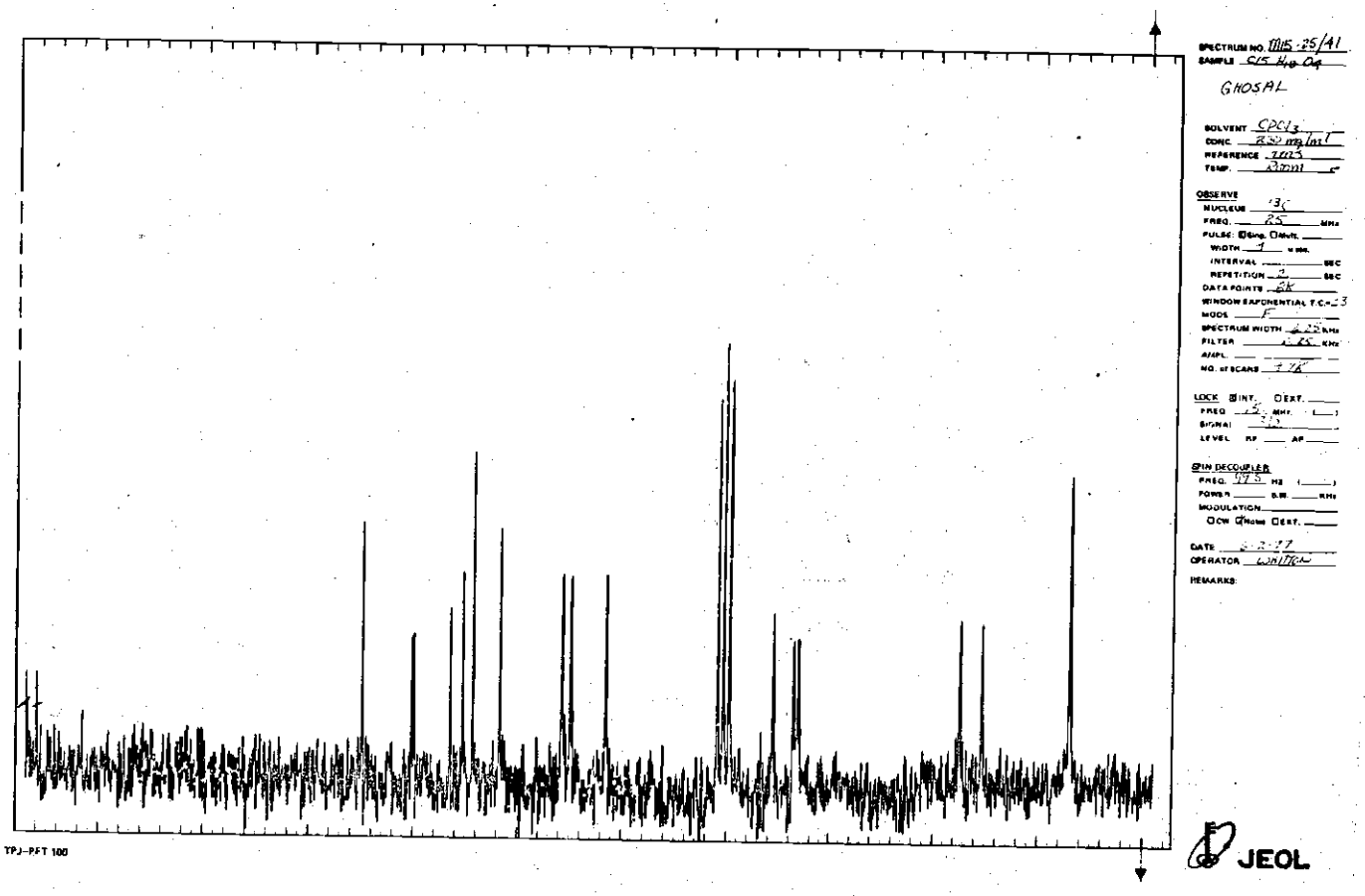


FIGURE 7. ^{13}C SPECTRUM (BROAD BAND DECOUPLED) OF RUGOSUMOL

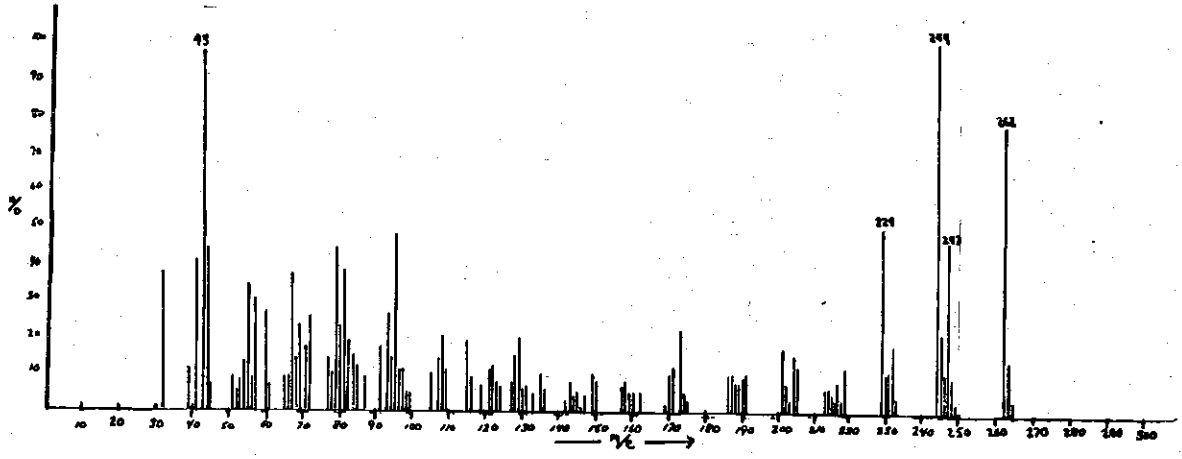


FIGURE 8. MASS SPECTRUM OF RUGOSUMOL

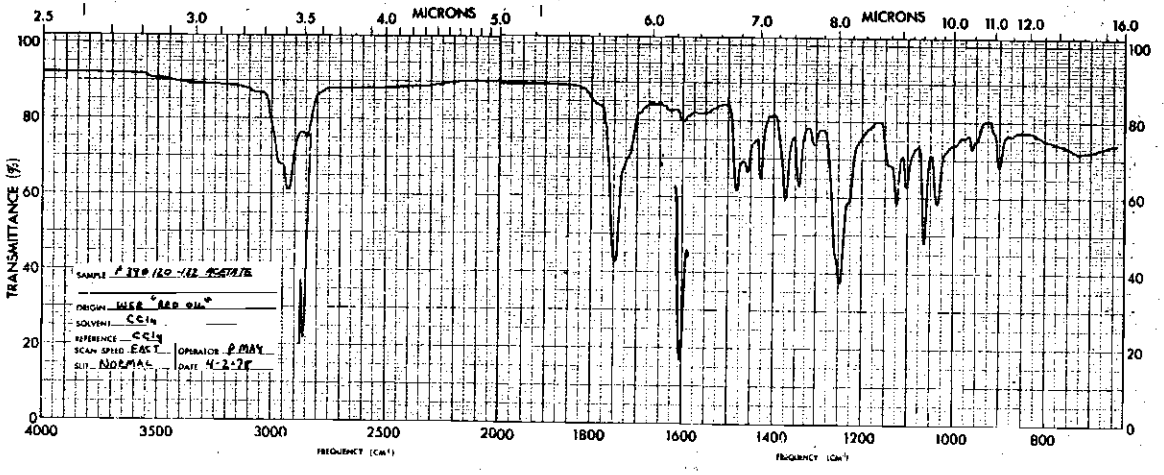


FIGURE 9. IR SPECTRUM (CCl_4) OF RUGOSUMOL ACETATE

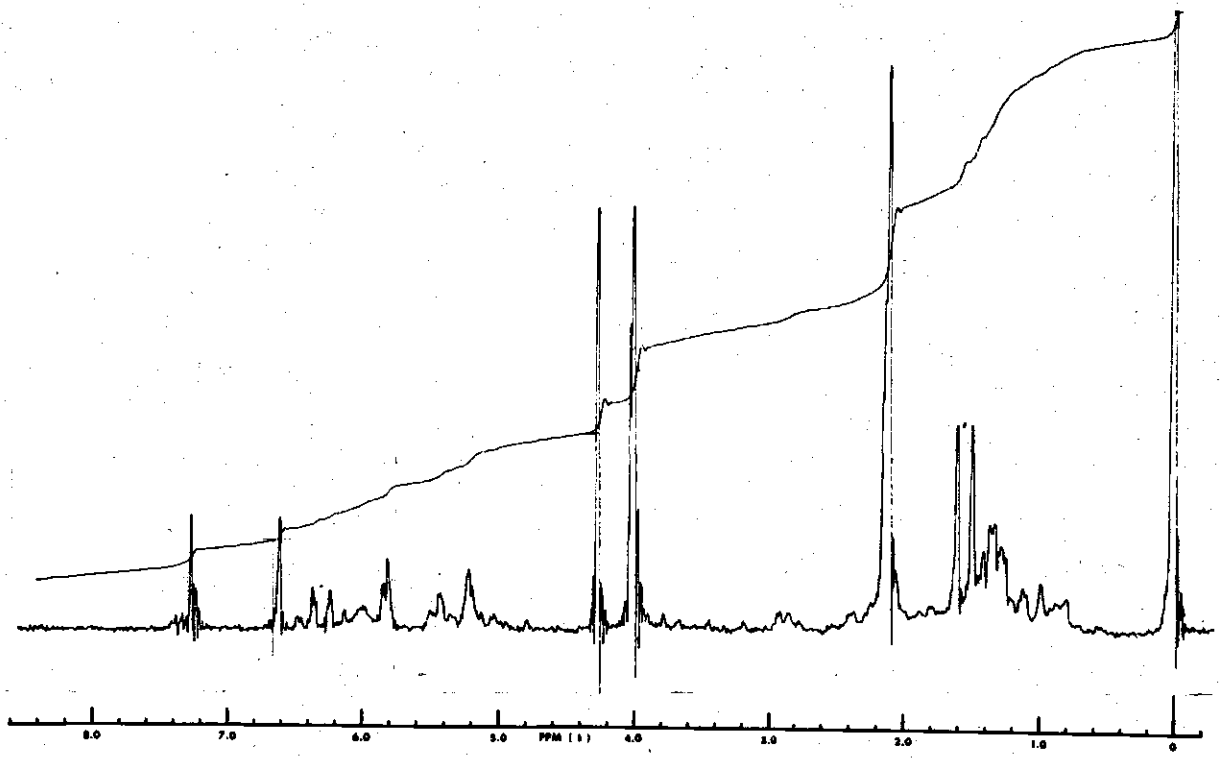


FIGURE 10. ^{1}H NMR SPECTRUM (60 MHz, $CDCl_3$) OF RUGOSUMOL ACETATE

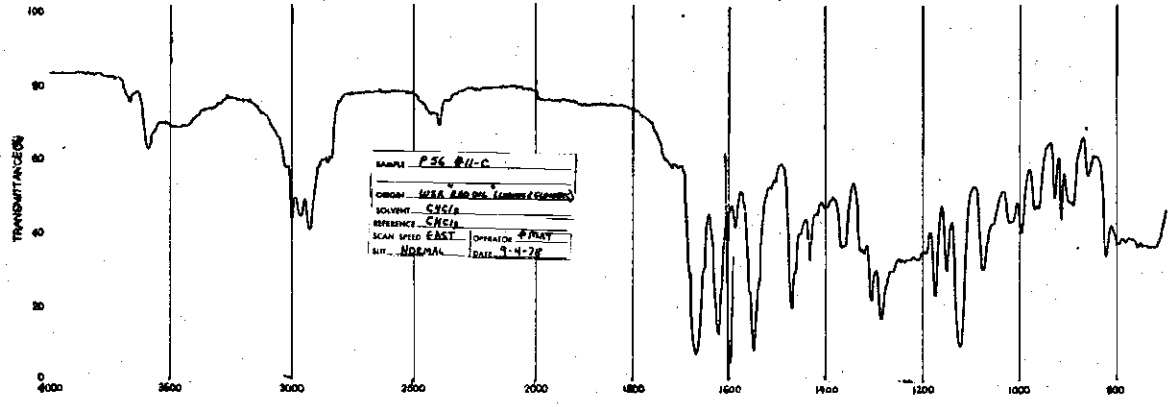


FIGURE 11. IR SPECTRUM ($CHCl_3$) OF RUGOSUMONE

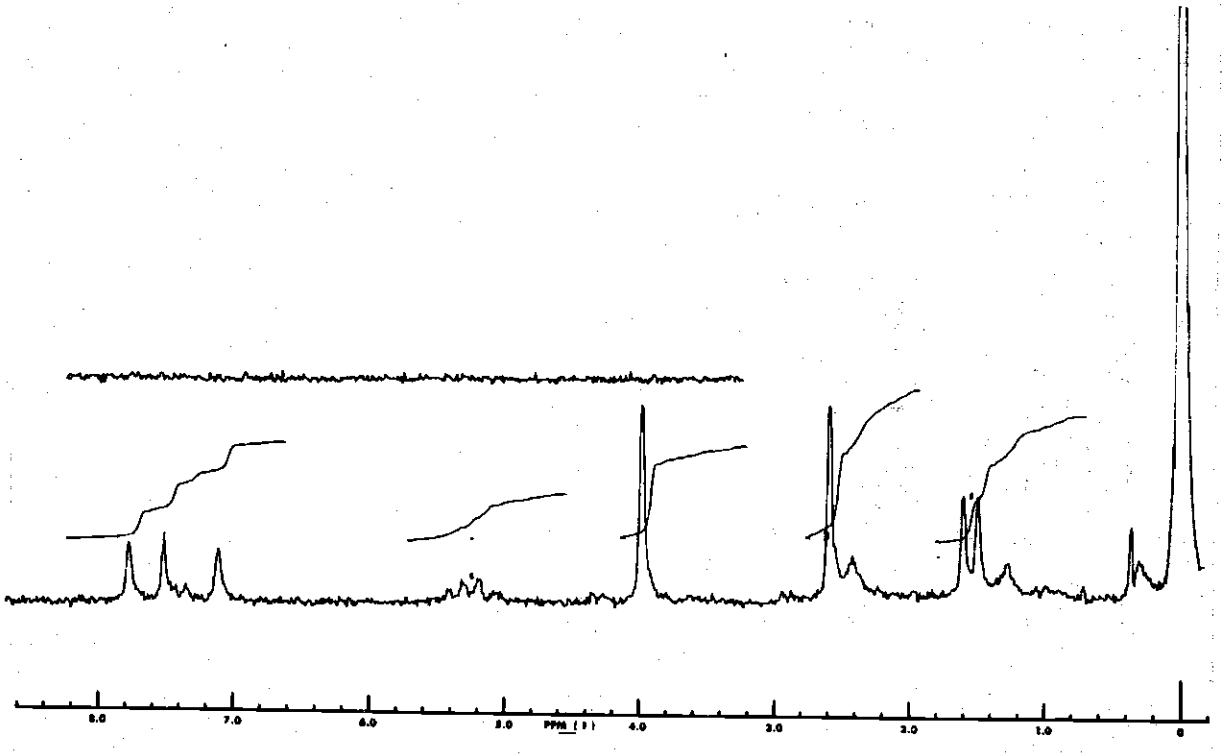


FIGURE 12. ^{1}H NMR SPECTRUM (60 MHz, $CDCl_3$) OF RUGOSUMONE (UPPER TRACE OFFSET 300 Hz)

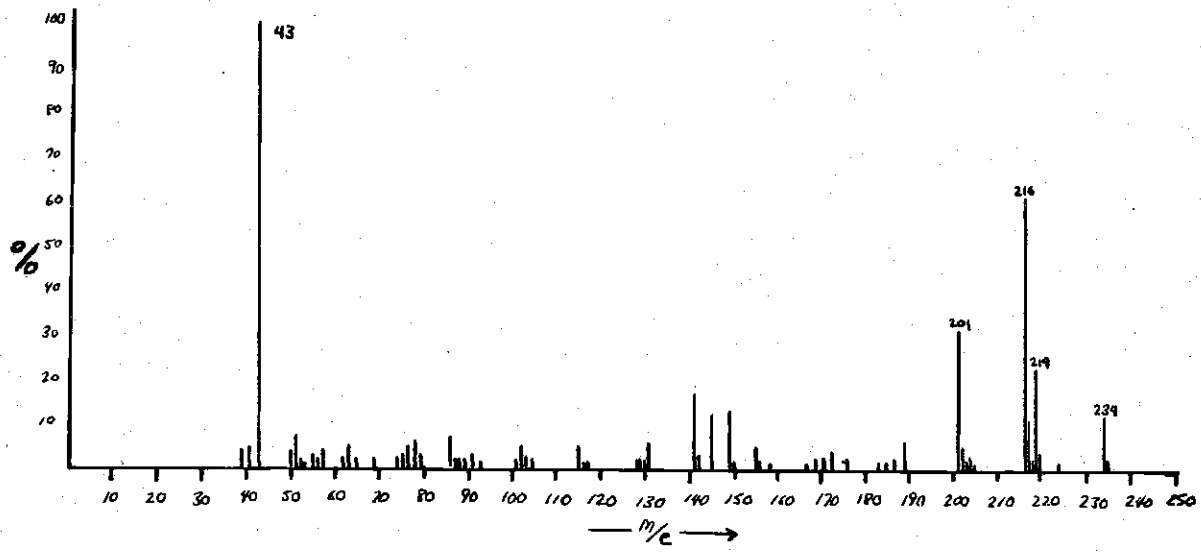


FIGURE 13. MASS SPECTRUM OF RUGOSUMONE

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VITA

Peter Edward May, eldest son of Mr. and Mrs. James E. May, was born July 30, 1952, in Corpus Christi, Texas. He was raised in Atlanta, Georgia, and attended Our Lady of the Assumption School and St. Pius X Catholic High School.

Mr. May attended the University of Georgia in Athens, Georgia from which he received the degree of Bachelor of Science with a major in Chemistry in March 1976. He began graduate study in the School of Chemistry at the Georgia Institute of Technology, Atlanta, Georgia, in September 1976.